PRECIPITATING AUTO-ANTIBODIES IN THE SERUM OF PATIENTS WITH CONNECTIVE TISSUE DISEASES

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INTRODUCTION

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'Horror autotoxicus'

The applications of the principles of immunity, the defence mechanism whereby an individual responds to the invasion of pathogenic bacteria, have been practised since the end of the eighteenth century. The response to this invasion has been shown to include the development of antibodies to these bacteria. These antibodies, which may frequently be detected by various techniques, in the serum of the individual, react with the pathogenic bacteria or their toxic products either to diminish their harmful effects or to participate in their destruction.

Not only have antibodies been shown to be produced against pathogenic bacteria, but the parenteral introduction of various substances, particularly proteins, into an individual may give rise to antibodies which react specifically with the substance introduced. This immunity mechanism has long been known to be able to distinguish between normal body constituents and foreign or heterologous material. Thus in immunological experiments in goats Ehrlich and Morgenroth (1900) found that the injection of red blood cells from one goat into another would give rise to antibodies causing lysis of the red cells of the donor and certain other goats, but not of the recipient's red cells; accordingly they formulated the principle of horror autotoxicus. This stated that no individual could react to produce antibodies to his own normal body

constituents and they believed that the formation of such tissue auto-antibodies would constitute a greater danger to the individual than any exogenous injury.

Evidence for auto-immunization

Evidence for the occurrence of auto-antibodies has, however, accumulated, showing that exceptions to the principle of horror autotoxicus are encountered. Thus Donath and Landsteiner (1904) demonstrated, in paroxysmal cold haemoglobinuria, a haemolysin which had the character of an immune body which was activated by cold to unite with the patient's own red blood cells but at internal body temperature of 37°C behaved as an autohaemolysin owing to union with complement. Later, Widal, Abrami and Brulé (1908), Chauffard and Troisier (1908) and Chauffard and Vincent (1909) observed autoagglutinins and autohaemolysins active at 37°C. in other types of haemolytic anaemia.

Subsequently many workers demonstrated haemolytic anaemia occurring in animals injected with anti-red blood cell serum produced in a different species. Among these, Dameshek and Schwartz (1938) produced haemolytic anaemia experimentally in guinea pigs by injection of the serum of rabbits which had been immunized against guinea pig red blood cells. These authors concluded, however, from comparison of their experimental work with certain types of naturally-occurring haemolytic

anaemia in man, that the latter were caused by auto-antibodies which had been adsorbed on to the red cells. The description of the anti-globulin reaction (Coombs test) by Coombs, Mourant and Race (1945, 1946), permitted further advances to be made in the study of antibodies to red cells. Boorman, Dodd and Loutit (1946) applied the direct Coombs test in five cases of acquired haemolytic anaemia and showed in-vivo sensitization of the patients' red cells by a gamma-globulin component of human serum and from these facts they reasoned that the acquired form of haemolytic anaemia was due to auto-antibody formation. Wiener, Battey, Cleghorn, Marson and Meynell (1953) detected, in the serum of a patient with haemolytic anaemia, an antibody which was directed against a single antigen on the patient's erythrocytes and failed to react with other human red blood cells which lacked this blood group factor. It has since been shown that antibodies occurring in the serum in acquired haemolytic anaemia are frequently directed specifically against one or more of the patient's own erythrocyte 'isoantigens'. The experimental production of auto-antibodies to red cells in animals has not yet been achieved. Bielschowsky, Helyer and Howie (1959) have reported in a strain of inbred mice a naturally-occurring haemolytic anaemia in which the erythrocytes were found to be coated with gamma-globulin and evidence that this was an auto-immune condition was confirmed by Holmes,

Gorrie and Burnet (1961), who transmitted the disease from affected adult mice to normal young mice of the same strain by transfer of splenic cells.

Autoantibodies in man have also been demonstrated to leucocytes and platelets. The demonstration of these antibodies is technically more difficult than the detection of antibodies to red cells, for which many reliable techniques have been elaborated. Walford (1960) reviewed many reports of the detection of leucocyte auto-agglutinins and dismissed a number of these on the grounds that there was insufficient evidence that auto- rather than iso-immunization had occurred. Killmann (1957) first put forward the clearest evidence for the occurrence of leucocyte auto-agglutinins in a patient with acute monocytic leukaemia and subsequently there have been several convincing reports. Auto-antibodies to platelets have been demonstrated in serum from patients with idiopathic thrombocytopenic purpura by Harrington, Sprague, Minnich, Moore, Aulvin and Dubach (1953) and by Stefanini, Plitman, Dameshek, Chatterjea and Mednicoff (1953).

Experimental auto-immunity

In recent years, various diseases have been produced experimentally in animals injected with tissue extracts or other tissue constituents incorporated into Freund's adjuvant (Freund and McDermott, 1942). Several

groups of workers have used either the animal's own individual (i.e. autologous) tissue, or tissues from other animals of the same species (homologous tissues), and have injected them into subcutaneous, intradermal or intramuscular sites. By this technique, lesions have been produced in the tissue or organ corresponding to that injected. Both the clinical and histological features of these diseases have, in some cases, borne such close similarities to certain naturally-occurring human diseases that an immunological mechanism has been postulated as the cause of the disease in man. In 1949, Kabat, Wolf and Bezer described the production of disseminated encephalomyelitis in rhesus monkeys by injection with autologous tissue incorporated in Freund's adjuvant: comparisons were drawn between this experimental disease and acute demyelinating encephalitis occurring naturally in man and occasionally in individuals who have received a series of injections of rabies vaccine which consists of a saline suspension of brain or desiccated spinal cords of infected rabbits. Eye lesions, both of the lens (Müller, 1952) and the uveal tract (Collins, 1949), and lesions of the testis (Freund, Lipton and Thomson, 1953), peripheral nervous system (Waksman and Adams, 1955), thyroid (Witebsky and Rose, 1956) and adrenal (Colover and Glynn, 1958) have all been induced experimentally in animals by injection of the corresponding, homologous antigen. Accordingly, diseases in which comparable lesions in the corresponding human organ occur

have been attributed somewhat speculatively to an auto-immune mechanism.

Auto-immunity to thyroid tissue

The occurrence of auto-antibodies to tissue cells and constituents is, in general, difficult to prove. Claims, in recent years, to have demonstrated auto-antibodies which reacted with various human tissues remain, in many cases, unsubstantiated. In some of these accounts there is no convincing evidence that the reactions concerned are of an immunological nature. In other reports, antibodies have been demonstrated, but it has not been possible to use the patient's own tissue antigens for the investigation and consequently it is not known whether the antibodies developed as a result of auto-immunization or whether they indicate only that iso-immunization has occurred. Of the various tissues against which auto-antibodies have been claimed to develop, evidence is most strong for the thyroid gland.

The interest in the occurrence of auto-immunity in thyroid disease was aroused by two almost simultaneous reports, the first on the experimental work in rabbits reported by Witebsky and Rose (1956) and the second on the serological investigations in naturally-occurring human chronic thyroiditis carried out by Roitt, Doniach, Campbell and Hudson (1956).

Witebsky and Rose described the production in rabbits of antibody

to rabbit thyroglobulin. The animals were injected intradermally with a crude saline extract of rabbit thyroid gland, or with "purified" rabbit thyroglobulin, incorporated into Freund's adjuvant, the purpose of the adjuvant being to increase the immune reaction. Following these injections, antibody to thyroglobulin was demonstrated in the serum of some of the animals by various techniques, including complement fixation, precipitation, and the tanned red cell agglutination technique of Boyden (1951). Since the antibody to thyroglobulin reacted with the autologous (i.e. the injected animal's own) thyroid tissue extract and since injection of the autologous thyroglobulin stimulated the development of the antibody, these experiments provided good evidence that auto-immunization had been effected. In some of the animals the production of antibody was accompanied by the development of chronic inflammation in the animal's own thyroid gland, these appearances being regarded as similar to naturally-occurring human chronic thyroiditis.

Evidence that auto-immunization plays a part in human chronic thyroiditis was provided by Roitt, Doniach, Campbell and Hudson in 1956. Serum from patients with Hashimoto's thyroiditis had previously been shown to contain high levels of gamma-globulin and to give abnormal reactions in serum-flocculation tests (Luxton and Cooke, 1956). This led Roitt and his colleagues to postulate that antibodies were being produced against the thyroid, and they succeeded in showing that

precipitating antibodies, which reacted with saline extracts of human thyroid tissue and with "purified" human thyroglobulin, occurred in the serum of patients with Hashimoto's thyroiditis.

There have since been found other types of circulating antibody in patients with varying degrees of chronic thyroiditis. The precipitin test, which was used at first by Roitt et al., has been shown to be positive only in patients with severe chronic thyroiditis. In Hashimoto's thyroiditis, the serum of about 70% of patients gives a positive precipitin result; in primary hypothyroidism, in which the thyroiditis is less florid than in Hashimoto's thyroiditis, about 14% of patients are positive. In patients with other thyroid diseases, such as thyrotoxicosis, the precipitin test is negative unless thyrotoxicosis is accompanied by severe chronic thyroiditis. The fluorescent antibody technique of Coons, Creech, Jones and Berliner (1942) has been applied in thyroid disease and precipitin positive sera have been shown to stain the thyroglobulin-containing colloid in frozen sections of thyroid gland (White, 1957).

Other, more sensitive techniques have been used to demonstrate antibody to thyroglobulin in human thyroid disease. The latex fixation test (developed by Hyland Laboratories, Los Angeles) is slightly more sensitive than the precipitin test (Anderson, Buchanan, Goudie and Gray) 1962); the tanned red cell agglutination method (Roitt and Doniach, 1958) is extremely sensitive and demonstrates thyroglobulin antibodies

not only in severe chronic thyroiditis, where these antibodies are generally present in high titres, but also in other thyroid diseases where there is often only a slight degree of thyroiditis accompanied by low concentrations of the antibody. Complement fixation tests, using as antigen extracts of thyrotoxic tissue, have demonstrated the presence of a second antibody to thyroid tissue which reacts with an antigen present in the microsomal fraction of thyroid epithelial cells and particularly abundant in extracts of thyrotoxic glands (Belyavin and Trotter, 1959). Pulvertaft, Doniach, Roitt and Hudson (1959) and Irvine (1960) reported a serum factor, in patients with chronic thyroiditis, which is cytotoxic to trypsinized thyroid epithelial cells grown in tissue culture. Goudie and McCallum (1962) have provided evidence that this factor is an antibody and that, like the thyrotoxic complement-fixing antibody, it reacts with thyroid epithelial microsomes and requires the presence of complement for its cytotoxic action. Another thyroid antibody, demonstrable by the fluorescent antibody technique and reacting with a constituent of the thyroid other than thyroglobulin, has been reported by Balfour, Doniach, Roitt and Couchman (1961).

Witebsky's production of experimental auto-immune thyroiditis in animals, taken together with the demonstration of various circulating antibodies to thyroid in patients with chronic thyroiditis, strongly suggests that auto-immunization may be the cause of thyroiditis in man.

However, none of the various antibodies in the human disease correlates closely with the extent of chronic thyroiditis, and none of them has been shown to be the agent responsible for destructive changes in the thyroid gland. It may be that this agent is a cell-bound antibody of the type which causes a delayed hypersensitivity reaction. Witebsky, Rose and Shulman (1956) carried out skin tests with thyroid extracts in auto-immunized rabbits and demonstrated a reaction of the delayed type. Skin tests in man were also carried out by Buchanan, Anderson, Goudie and Gray (1958), but the reaction observed was believed to be a delayed Arthus phenomenon. The results of both these groups of workers could be attributed to circulating, rather than cell-bound, antibodies; true delayed hypersensitivity would be difficult to demonstrate as it would tend to be obscured as a result of the initial reaction due to circulating antibodies. Passive transfer of immune serum has been unsuccessful in producing auto-immune thyroiditis (Roitt and Doniach, 1958) but recently Felix-Davies and Waksman (1961) have reported that transfer of lymph nodes or splenic cells from guinea pigs immunized against thyroid tissue has produced experimental thyroiditis in the recipient guinea pigs. It therefore appears that the destructive agent in experimental thyroiditis is a cell-bound, rather than a circulating antibody and it is possible that this may also be true for human chronic thyroiditis.

Auto-immunity to other tissues

Evidence for auto-antibodies occurring in other diseases has also been strengthened in recent years. In patients with idiopathic Addison's disease antibodies to adrenal tissue have been demonstrated by complement fixation and fluorescent antibody tests (Anderson, Goudie, Gray and Timbury, 1957, Blizzard, Chandler, Kyle and Hung, 1962). Antibodies to colon have been demonstrated in serum from patients with ulcerative colitis (Broberger and Perlmann, 1959) and in serum from patients with pernicious anaemia antibodies have been demonstrated reacting with extracts of gastric mucosa (Irvine, Davies, Delamore and Williams, 1962, Taylor, Roitt, Doniach, Couchman and Shapland, 1962, Markson and Moore, 1962).

Immunological associations of Sjögren's syndrome

The appearance of the thyroid in Hashimoto's thyroiditis is one of extensive infiltration by lymphocytes and plasma cells, associated with large, eosinophilic epithelial cells, forming small thyroid follicles, or arranged in small groups with no remaining follicular structure. Cardell and Gurling (1954), reviewing three cases of Sjögren's syndrome, characterized by degenerative and atrophic changes in the lacrimal and salivary glands and accompanied by decreased secretion of these glands, were struck by the resemblance of these lesions to those occurring in the thyroid gland in Hashimoto's thyroiditis and in primary hypothyroidism. The resemblance between Hashimoto's thyroiditis and Sjögren's syndrome was

further emphasized by Heaton (1959), who stated that the two have in common age and sex incidence, raised erythrocyte sedimentation rate, increased gamma-globulins, and abnormal thymol-turbidity tests; as a result of these associations, he postulated that Sjögren's syndrome also had an auto-immune basis. Jones (1958) examined serum from 40 patients with Sjögren's syndrome and reported that there was in one patient a serum precipitating factor which reacted with extracts of human salivary and lacrimal glands and also with some extracts of kidney tissue; he also stated that the serum gamma-globulins were raised in this patient but did not state whether the precipitating factor was in the gamma-globulin serum fraction and therefore likely to be an immune antibody.

The frequent occurrence of various degrees of thyroiditis in Sjögren's syndrome has been referred to by Heaton (1959), and Bunim (1961), in a review of 40 cases of Sjögren's syndrome, detected antibodies to thyroglobulin in eleven.

In addition to its similarities to and associations with chronic thyroiditis, Sjögren's syndrome is also associated with the group of conditions termed "diffuse collagen diseases" by Klemperer, Pollack and Baehr (1942) and now more widely termed "connective tissue diseases." Included in this group of conditions are rheumatoid arthritis, systemic lupus erythematosus, chronic discoid lupus erythematosus, progressive systemic sclerosis, dermatomyositis and polyarteritis nodosa. The

commonest association between Sjögren's syndrome and the connective tissue diseases is provided by the high incidence of rheumatoid arthritis in patients with Sjögren's syndrome. Heaton (1959) revieved a series of twenty-eight patients with Sjögren's syndrome and described many clinical and pathological features commonly observed in systemic lupus erythematosus. The association of Sjögren's syndrome with systemic lupus erythematosus and with progressive system sclerosis has been noted in several recent reports, e.g. Bunim, 1961, Shearn, 1960, 1961.

PERSONAL INVESTIGATIONS

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In view of the above features and associations of Sjögren's syndrome, the serum of patients with this condition was tested for the presence of auto-antibodies. From the work of Jones (1958), referred to above, and from analogy with Hashimoto's thyroiditis, it was anticipated that auto-antibodies reacting specifically with salivary and lacrimal tissues might be detectable. This expectation was not fulfilled, since, although auto-antibodies were detected in Sjögren's sera, they reacted not only with extracts of lacrimal and salivary tissue but also with various other tissue extracts.

As a result of these findings, serum was also examined from patients with other connective tissue diseases, using the same techniques, and in addition to the auto-antibodies previously demonstrated in Sjögren's sera, other antibodies were also detected. In addition to these investigations, the associations between chronic thyroiditis, Sjögren's syndrome and the connective tissue diseases led to the examination of the serum of patients with the latter two types of disease for antibodies to thyroglobulin, using a sensitive technique. The work to be described thus falls into three sections.

Section I. Serum from patients with Sjögren's syndrome was examined for antibodies reacting with human lacrimal and salivary tissue extracts.

Section II. The serum of patients with other connective tissue diseases,

including systemic lupus erythematosus, chronic discoid lupus erythematosus, progressive systemic sclerosis, dermatomyositis, rheumatoid arthritis and polyarteritis nodosa was examined for antibodies reacting with extracts of a wide variety of human tissues and organs.

Section III. The incidence of antibodies to thyroglobulin, as detected by the tanned cell agglutination technique, was estimated in the serum of patients with connective tissue diseases, including Sjögren's syndrome.

SECTION I

TESTS FOR PRECIPITATING ANTIBODIES TO SALIVARY AND LACRIMAL GLAND EXTRACTS IN THE SERUM OF PATIENTS WITH SJOGREN'S SYNDROME

The method used by Jones (1958) to demonstrate a precipitating factor in Sjögren sera was a modification of the technique of Ouchterlony (1953) in which immune serum and antigen are placed in the wells in an agar plate and the precipitating antibody and antigen diffuse into the intervening agar, where they react to form a visible precipitate. Jones used a sloppy agar and an elaborate arrangement of troughs in the agar; troughs containing the undiluted test sera were refilled as it was found that the serum factor (? antibody) was weak. On the other hand, the tissues were extracted with 2 or 5 volumes of saline solution and it was not considered necessary to refill the troughs containing the extracts, suggesting that the tissue precipitating factor (? antigen) concerned was relatively abundant.

In an attempt to repeat Jones' observations, serum was obtained initially from 2 cases of Sjögren's syndrome and was tested in agar-gel diffusion plates using, as antigen, extracts of human salivary or lacrimal tissue. In the light of previous experience of precipitation reactions involving antibody to thyroglobulin, a somewhat different modification of the Ouchterlony technique was adopted (see p. 143) in preference to that used by Jones. Initially, a 50% extract of parotid

gland in 0.9 % saline solution (p.141) was used both undiluted, and at a dilution of 1 in 4, whereas the serum was used undiluted. In addition to the 2 specimens of Sjögren serum, 5 control sera, obtained from patients with other conditions, were also tested with both concentrations of parotid gland extract. These tests were examined daily; after 2 days a band of precipitate was observed between two of the wells, one of which contained undiluted parotid extract and the other contained a serum from a patient (Tra) with Sjögren's syndrome (Fig. 1). This precipitate was situated very close to the well containing the tissue extract. The other sera did not react to form a precipitate over a period of several days.

This encouraging result, which showed the presence in serum Tra of a factor which reacted with a constituent of salivary gland extract to produce a precipitate, led to this serum being singled out for further investigation.

Reactions of serum Tra with extracts of various human tissues and blood constituents.

Various tissue extracts, including lacrimal gland, 3 parotid glands, 4 submandibular glands, 2 thyrotoxic thyroids, kidney, liver, adrenal and normal thyroid, were tested in Ouchterlony agar-gel diffusion plates around undiluted serum Tra. All these tissue extracts appeared to react to form a precipitate with serum Tra although in many cases a very

dense zone of opacity, or halo, round the wells containing the tissue extract, made the band of precipitate rather difficult to discern. (Fig. 2). A positive result with serum Tra was also obtained with extracts of brain (both white and grey matter), spleen and testis. Thyrotoxic thyroid extract appeared to give the most distinct band of precipitate and the least opacity around the tissue extract well. Jones had described opacity occurring around the serum well in his Ouchterlony tests and had found that the method described by Halbert, Swick and Sonn (1955) of incorporating glycine into buffered agar diminished this effect. I prepared glycine-buffered agar, as Jones had done, in order to try to minimize the haloes around the wells containing the tissue extracts. This made no difference, however, the haloes around the wells in the buffered glycine-saline agar being as dense as those in plates containing azide-saline agar.

Titration of precipitating factor in serum Tra.

A series of fourfold dilutions of serum Tra in 0.9 % saline solution was tested with a thyrotoxic thyroid gland extract, used undiluted; a precipitate was observed up to a dilution of 1 in 256 of serum Tra. With undiluted thyrotoxic thyroid gland extract, serum Tra gave distinct, straight lines of precipitate at all dilutions from 1 in 1 to 1 in 256. The position of the precipitate varied from very close to the tissue extract well with undiluted serum, to a position which was slightly

nearer the serum well than the tissue extract well with the higher dilutions (Figs. 3a and 3b). The optimal dilutions of serum Tra (i.e. the dilutions at which the bands of precipitate were most sharply defined and approximately mid-way between the wells) were between 1 in 16 and 1 in 64. Therefore in most of the agar-gel diffusion tests performed subsequently with serum Tra, the dilution used was 1 in 32.

In further tests, in which various sizes of wells were spaced at various distances from each other, it was found that wells of 10 mm. diameter, with their centres 15 mm. apart, gave optimal precipitation.

The incidence of the precipitating factor reacting with serum Tra in various tissues and body fluids.

Tissues obtained post-mortem from one individual, including liver, kidney, adrenal, submandibular and thyroid gland, were extracted and tested against dilutions of serum Tra and dilutions of a control (Hashimoto) serum containing precipitating antibody to thyroglobulin: serum Tra reacted up to a dilution of 1 in 256 with every extract, whereas the control serum was negative with all except thyroid extract. Suspensions of whole blood, washed red blood cells (lysed by repeated freezing and thawing), platelets, serum and pooled gamma-globulin prepared by the Scottish National Blood Transfusion Association, were tested in various dilutions with a 1 in 32 dilution of serum Tra; no precipitation was observed in any of these tests. When leucocyte

extracts were tested with serum Tra, variable results were obtained. Leucocyte extracts from normal individuals and from patients with chronic myeloid leukaemia failed to give a precipitate with serum Tra, but leucocyte extracts from patients with lymphatic leukaemia gave a positive result. This finding with leucocytes from different sources will be discussed more fully on p.27. Tissues of low cellularity, such as joint fluid, costal cartilage, skeletal and heart muscle and umbilical cord, were also tested with serum Tra; no precipitation was detected with any of these tissues. Skin fibroblasts, (maintained for 7 months in tissue culture and kindly supplied by the Department of Genetics, Glasgow University) were obtained from 4 individuals and extracted; all 4 extracts were negative with serum Tra. Extracts of pooled spermatozoa from several individuals were also negative. Saliva and tears, the secretion of which is considerably reduced in Sjogren's syndrome, were obtained from blood group O individuals, tested unconcentrated with serum Tra and found to be negative. A solution of thyroglobulin (5 mg/cc) prepared by the method of Derrien, Michel and Roche (1948) and shown to react with a serum containing antibody to thyroglobulin, failed to react to form a precipitate with serum Tra. The precipitating factor in serum Tra is therefore not anti-thyroglobulin.

Titration of tissue extracts reacting with serum Tra.

When dilutions of tissue extracts were titrated with serum Tra, diluted 1 in 32, the highest dilution which produced a precipitate was 1 in 8, and this was exceptional. Most tissue extracts reacted only when undiluted, or when diluted 1 in 2 or 1 in 4.

The precipitating factor in serum Tra.

Immunoelectrophoresis. Immunoelectrophoresis of serum Tra was carried out in agar as described on p. 147. This showed that the precipitating factor in serum Tra had the electrophoretic mobility and pattern of gamma-globulin (Fig. 4).

Ultracentrifugation. A quantity of serum Tra was sent to Dr. P.A. Charlwood (of the National Institute for Medical Research, Millhill, London) who kindly carried out ultracentrifugation of the serum. The method used was a modification of the sucrose density-gradient method in which radioactive-labelled gamma-globulin of known sedimentation constant was added to serum Tra to act as a tracer. The fractions of serum Tra provided by Dr. Charlwood were tested in agar-gel diffusion tests and the precipitating factor in serum Tra was found to be present only in the fractions containing the 7S-gamma-globulin fraction of the serum.

The results obtained by immunoelectrophoresis and ultracentrifugation of serum Tra provide good evidence that the serum factor which is concerned

is an antibody.

Direct tests for identity: comparison of antigens.

Human tissue extracts reacting with serum Tra were compared by means of the Ouchterlony agar-gel diffusion test for identity of antigen-antibody systems. Pairs of extracts were compared by placing them in 10 mm. diameter wells situated with edges 3 mm. apart, and serum Tra was placed in a well of 6 mm. in diameter as shown in Fig. 5. As this last well was smaller than that previously shown to give optimal precipitation with a 1 in 32 dilution of serum Tra, this was compensated for by using serum Tra at a dilution of 1 in 10 in this experiment. The arrangement of the wells was such that the factors present in the extracts under comparison would react with serum Tra. to form precipitates which would meet each other, either to fuse completely, indicating a "reaction of identity" of the tissue antigens or to cross each other, suggesting a "reaction of non-identity" or to fuse partially with spur formation indicating a "reaction of partial identity" of the tissue precipitating factors. These patterns of precipitation, described by Ouchterlony (1953), have been shown to be considerably altered by variations in concentrations of the reagents (Korngold, 1956, Wilson and Pringle, 1956), However, when extracts of liver, kidney, adrenal, submandibular and thyroid gland from one individual, all shown to give a distinct

precipitate with serum Tra., were set up in pairs, kidney with liver, kidney with thyroid, thyroid with submandibular, etc., all these organ extracts were observed to give lines of precipitate which fused completely with each other, indicating "complete identity" of the tissue precipitating factors. Extracts of human tissues from other individuals also gave reactions of identity with this set of extracts. Extracts of lymphatic leukaemic leucocytes also gave reactions of complete identity with these organ extracts.

It is thus concluded that the reactions of serum Tra with a wide variety of human tissue extracts are due to a single tissue antigen present in all the extracts. This antigen is also present in extracts of lymphatic leukaemic leucocytes and is therefore regarded as a cellular constituent. Because it reacted well with serum Tra, gave minimum halo-effect, and was readily obtainable in the fresh state, thyrotoxic thyroid tissue was used subsequently, except when otherwise stated, in tests with serum Tra.

Annulment experiments.

Attempts to annul the precipitating antibody in serum Tra by admixture with tissue extracts. The previous section, reporting the reactions of identity of tissue extracts, provides a means of comparing the precipitating factor in the extracts. Another method by which the extracts may be compared is by treating serum Tra with a relatively large volume of a

tissue extract and testing the resulting product for precipitating antibody with various tissue extracts, including the one used for absorption. If the antibody in the mixture has been annulled by the tissue extract, it would not be expected to form a precipitate with the extract used to annul it, or with any other tissue extract containing the same tissue antigen

Accordingly, a mixture was prepared containing one part of serum Tra and fifteen parts of thyroid extract. Similar mixtures of serum Tra with extracts of liver, kidney, adrenal and parotid gland were prepared, and all were kept overnight at 4°C. to allow antigen-antibody reaction to occur. The following day, each mixture was tested in an Ouchterlony agar-gel diffusion plate with each of the tissue extracts and also with serum Tra. All the mixtures of serum Tra with tissue extract reacted with the various tissue extracts including the extract used to annul the serum. The same mixtures, however, failed to react with suitable dilutions of serum Tra. It therefore appeared that in the mixtures the tissue extract antigens were annulled by serum Tra. Since annulment of the antibody in serum Tra by tissue extract antigens had not been demonstrated, further experiments were performed in which serum Tra was mixed with greater volumes of tissue extract, but even when the final dilution of serum Tra in the mixture was 1 in 100, serum Tra continued to react to give a precipitate with some of the tissue extracts used for

absorption.

This attempt to annul serum Tra with tissue extract in order to show the identity of the precipitating factors in the tissue extracts was therefore unsuccessful. It showed, however, that the tissue extracts contained very low concentrations of antigen relative to the amount of antibody in serum Tra.

Annulment of tissue precipitating factor.

Serum Tra was mixed with tissue extract in the ratio of one part of serum to nine parts of extract. A normal serum was mixed in the same proportions with the tissue extract, this mixture serving as control for the activity of the tissue precipitating factor. Both mixtures were left overnight at 4°C. and on the following day were tested in agar-gel diffusion plates with serum Tra (1 in 32), and with the tissue extract used for absorption. Tissue extracts which had been treated in this manner with serum Tra failed to form a precipitate when tested with serum Tra ; precipitation was not inhibited by similarly treating the tissue extracts with normal serum. The mixtures containing serum Tra. continued to react with tissue extract to form a precipitate, showing that excess of antibody was present in the mixture. These results show that the antigenic constituent of tissue extracts which react with serum Tra is readily annulled by excess antibody, thus confirming that relatively small amounts of antigen are present in the extracts.

Tests of serum Tra with animal tissues.

Extracts prepared from animal tissues obtained immediately after death were tested in Ouchterlony plates with serum Tra (1 in 32). Precipitates were observed with extracts of the livers, kidneys and spleens of guinea pigs, rabbits and mice. Tissue extracts from rats caused a particularly dense zone of opacity around the wells in which they were placed, but, with the exception of one extract of rat spleen, no precipitates were observed between these extracts and serum Tra. Similar dense zones of opacity were observed when tissue extracts from hen and trout organs were tested against serum Tra, and no precipitates could be detected. Normal human serum, tested at several dilutions, gave no precipitates with any of these organs. Direct tests of identity were carried out as described previously (p. 22-23) and reactions of identity for serum Tra were obtained between extracts of human and animal tissues.

Yeast ribonucleic acid and deoxyribonucleic acid from calf thymus nuclei (Nutritional Biochemicals Corporation), tested in a wide range of concentrations, failed to give a precipitate with serum Tra.

Effect of heat on antibody in serum Tra and on tissue antigens.

Serum Tra was heated at 55°C. for 2 hours and was then tested with thyroid tissue extract. The precipitate obtained had the same appearance

as with unheated serum. Only faint precipitation occurred, however, in tests with serum Tra heated at 65°C. for 30 minutes.

The precipitate between serum Tra and tissue extract heated at 45°C. for 30 minutes was much fainter than with unheated tissue extract and with tissue extract heated at 55°C. for 30 minutes, no precipitate was obtained. Incubation of the tissue extract at 37°C. for about 6 hours also weakened its precipitating antigen.

Effect of enzymes on antigen for serum Tra.

An extract of thyrotoxic thyroid, buffered at pH 7.4, was treated with 0.2% crystalline trypsin (British Drug Houses), at 37°C. for 2 hours. This treatment was found to destroy the antigen for serum Tra. Crystalline chymotrypsin, used at concentrations of 0.4% and papain at concentrations of 0.1 and 0.2% also destroyed the antigen for serum Tra. 0.4% diastase solution had no effect on the antigen. Deoxyribonuclease (Nutritional Biochemicals Corporation) dissolved in phosphate buffer (pH 6.8), containing 5 uM $MgCl_2$ per ml., was added to thyrotoxic thyroid extract and the mixture was incubated for 1 hour at 37°C. as described by Deicher, Holman and Kunkel (1959). This treatment had no effect on the antigen for serum Tra: ribonuclease was also without effect on the antigen.

As previously stated (p. 20), the tissue antigen was present in all

extracts of lymphatic leukaemic leucocytes which were tested, but was not demonstrated in extracts of normal leucocytes or of leucocytes from patients with myeloid leukaemia. These results suggested that the myeloid leukaemic leucocytic extracts and the normal leucocytic extracts might contain a factor, possibly enzymic, which was responsible for the destruction of the antigen for serum Tra. Accordingly, equal volumes of thyrotoxic thyroid extract and myeloid leukaemic leucocytic extract were incubated at 37°C. for 3 hours and then tested for antigen for serum Tra in an agar-gel diffusion test. Similar mixtures of thyroid and leucocytic extracts were prepared and incubated, using an extract of normal leucocytes and an extract of lymphatic leukaemic leucocytes. The results obtained showed that the antigen was destroyed by incubation with extracts of myeloid leukaemic or normal leucocytes, but that a precipitate was still obtained when the antigen was mixed and incubated with an extract of lymphatic leukaemic leucocytes. A control, consisting of thyrotoxic thyroid extract, diluted with an equal volume of saline solution and incubated at 37°C. for 3 hours, still gave a precipitate with serum Tra. This finding suggests that the inhibitory substance present in normal and myeloid leucocyte extracts is of an enzymic nature.

Quantitative precipitin curve for serum Tra.

The quantitative characteristics of the precipitin reaction between

serum Tra and the corresponding antigen were studied in the following series of experiments.

Principle. In a classical antigen-antibody precipitin curve, as described by Heidelberger and Kendall (1935), precipitation occurs on addition of antigen to excess of antibody, the amount of precipitate depending on the amount of antigen present; thus, increasing the amounts of antigen added to a constant amount of antibody results in an increase in the amounts of precipitate formed, until a maximum is reached in the equivalence zone where neither antigen nor antibody is in excess. In the zone of antigen excess, soluble antigen-antibody complexes are formed and the amount of precipitate decreases in this region. Rabbit antibodies regularly form this type of curve, whereas with certain horse antibodies, inhibition of precipitation occurs with antibody excess as well as in the antigen excess zone.

Experiments.

1. Attempt to determine the amount of protein precipitated. The antigen used was a thyrotoxic thyroid gland extract. Both antigen and serum were centrifuged at 12,000 g. for 15 minutes to remove fine, particulate material and mixtures of the 2 reagents were prepared in tubes 2 x 0.6 cm. as shown.

Tube No.	1	2	3	4	5	6	7	8	9	10	11
Dilutions of serum Tra, (3 drops per tube)	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	Nil
Thyroid extract (3 drops per tube)	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1

A second row of tubes containing mixtures of undiluted thyroid extract and dilutions of a normal serum instead of serum Tra, was prepared at the same time. To each tube was added one drop of 1% sodium azide solution, to prevent bacterial contamination. Both rows of tubes were kept at room temperature during the day and at 4°C. overnight. No visible precipitation had occurred after 24 hours. The small precipitates obtained after 72 hours were washed 3 times with 0.9% saline solution at 4°C. and each was then dissolved in 0.1 M sodium carbonate solution. The amount of precipitated protein was so small that it was not possible to estimate it satisfactorily by measuring its absorption of ultra-violet rays at a wavelength of 280 mu. on a Uvispek spectrophotometer.

This preliminary attempt to prepare a quantitative precipitin curve for serum Tra having been unsuccessful, it was not thought worthwhile to continue with this method using larger volumes of reagents. The following

method of obtaining a precipitin curve for serum Tra was therefore used.

2. Radioactive labelling of thyroid extract with iodine (^{131}I). Labelling of protein with radioactive iodine (^{131}I) provides a sensitive technique for tracing small amounts of the labelled reagent. It is normally a satisfactory technique for use in immunological reactions, little alteration in the immunological properties of certain protein antigens being observed after labelling with small amounts of isotope. The method is essentially that described by Francis, Mulligan and Wormall (1954) and is given on p. 157.

15 ml. of a fresh, thyrotoxic thyroid gland extract was labelled by this procedure. On centrifugation of this extract at 34,000 g. for 1 hour after labelling, an appreciable deposit was formed. The supernate was pipetted off and tested for antigenicity in an agar-gel diffusion test. Serum Tra, diluted 1 in 25, formed a faint band of precipitate with this radioactive antigen, whereas the untreated extract had formed a well-defined precipitate. This result suggested that the antigen had been weakened by the treatment and dilution occurring during the process of radioactive labelling. It was decided, nevertheless, to test this antigen in a fluid medium. Accordingly, 10 drops of the radioactive thyroid gland extract were mixed with 2 drops of serum Tra; 10 drops of the thyroid gland extract were also mixed with 2 drops of a normal serum

and both tubes were kept at 4°C. for 48 hours. The small amounts of precipitate obtained after the tubes had been centrifuged at 1500 g. at 4°C. were washed 3 times at 4°C. with saline solution and resuspended in saline solution. The radioactivity present in the supernates, washings, and deposits was measured in a well-type scintillation counter, and the results are presented in Table 1.

These results show no significant difference in the radioactivity of the final deposits in the two tubes or in the washings. It was concluded that either radioactive labelling of the antigen for serum Tra had not been achieved or that the antigen had been largely destroyed during the process of ^{131}I - labelling. Deposition of radioactive material in the mixture containing normal serum suggests that some denaturation had occurred. The next attempt was made with ^{131}I - labelled serum.

TABLE 1

	Serum Tra + thyroid - ^{131}I Counts per 100 secs.*	Normal serum + thyroid - ^{131}I Counts per 100 secs.*
Supernate	520,800	520,500
First wash	60,000	52,000
Second wash	4,342	5,668
" deposit	5,668	7,113
Third wash	742	710
" deposit	4,316	5,994

*These results have been corrected by subtracting the counts per 100 seconds due to background.

3. Radioactive labelling of serum Tra. Labelling with ^{131}I was carried out on whole serum using the same method previously described for thyroid extract (p.157). 10 ml. of serum Tra was used for this purpose and 10 ml. of normal serum was labelled at the same time. The antigen used with the labelled serum was a pooled extract of twenty thyroids. Both the labelled serum and the pooled thyroid extract were centrifuged at 34,000 g. for 30 minutes before use. 2 ml. amounts of dilutions of thyroid extract were mixed with 1 ml. amounts of a constant dilution of ^{131}I-labelled serum Tra in tubes 7.5 x 1.25 cm. Two rows of tubes were set up in duplicate, the first series of tubes (rows A and B) contained serum Tra diluted 1 in 4 throughout; the second series (rows C and D) contained serum Tra diluted 1 in 64 throughout. To each tube was added 2 drops of 2% sodium azide solution. The tubes were kept at 4°C. for 48 hours and the precipitates were deposited by centrifugation at 4°C. and washed, as before, with cold saline solution. The supernatant solutions and some of the washing fluids were kept for radioactive counting and the precipitates were counted after each wash. In this way it was determined when most of the uncombined radioactivity had been removed by washing. A standard dilution of serum Tra was also made and counted at intervals: this control was prepared in order to make allowances for fluctuations which might occur in the well-type scintillation counter and in the mains electricity supply.

Series A and B. Each precipitate, thrice washed, was suspended in 2 ml. 0.1 M sodium carbonate solution and kept overnight at 4°C. It was then transferred to a clean tube and the original tube was rinsed out with a further 2 ml. of sodium carbonate solution which was added to the original 2 ml. volume containing the dissolved precipitate. The radioactive count-rate of the precipitate, now dissolved in 4 ml. 0.1 M sodium carbonate solution was then measured. The results are presented in Table 2 (p. 36) and are shown graphically in Fig. 6.

Considering that the precipitates were repeatedly washed before counting, the agreement in the two duplicate series is close. The results show that increasing the amount of antigen increased the amount of antibody precipitated. The supernates from series B, tubes 1 to 8, were tested for antibody excess by the Ouchterlony agar-gel diffusion technique; excess antibody was shown to be present in all 8 tubes of series B, confirming that insufficient antigen had been present in any of the mixtures to annul the antibody in serum Tra. Thus the region of antigen excess was not reached.

TABLE 2

Series A and B

Tube No.	Concentration of antigen added (%)	Dilution of ^{131}I-serum Tra added	Precipitate Counts per 100 secs.*	
			Series A.	Series B.
1.	100	1 in 4	19,350	18,250
2.	75	" " "	15,300	- †
3.	50	" " "	12,230	11,387
4.	33	" " "	7,610	9,065
5.	20	" " "	4,830	4,970
6.	14	" " "	3,510	3,456
7.	10	" " "	2,480	2,300
8.	5	" " "	758	250
9.	0	" " "	0	0
10.	20	Normal serum 1 in 4	-	-

† Not done

* These results have been corrected by subtracting the counts per 100 seconds due to background (300 counts per 100 seconds) and to the radioactive deposit in Tube 9 in which there was no thyroid extract (4,200 counts per 100 seconds in series A, 3,200 counts per 100 seconds in series B). The radioactivity occurring in Tube 9 is due to uncombined and probably denatured protein of serum Tra.

Series C and D. The results for series C and D, in which serum Tra was used at a dilution of 1 in 64, are shown in Table 3 (p. 39) and graphically in Fig. 7. Again, there is fairly close agreement between these two duplicate series. The equivalence zone, where the curve flattens out, is shown, and at the highest antigen concentration there is a diminution in the amount of antibody precipitated. The supernates 1 to 8 of series D were tested for antibody excess in agar-gel diffusion tests. Marked antibody excess, demonstrated by the formation of well-defined bands of precipitate with thyrotoxic thyroid gland extract, was shown only in the 3 tubes containing the lowest antigen concentrations (Tubes 5-7). Faint precipitates were seen with the next two higher antigen concentrations (Tubes 3 and 4) and no lines were detected when the supernates containing 100% (undiluted) and 50% antigen dilution (Tubes 1 and 2) were tested for antibody excess.

The results of the agar-gel diffusion tests on the supernates of series B and D show, as would be expected from the precipitin curves, that excess antibody was present in all tubes save those in row D containing the highest antigen concentrations. The antigen extract used in these tests was not very potent, and in series A and B the amount of serum Tra was too great to be annulled by the antigen present, therefore the zone of equivalence was not reached. In series C and D, in which serum Tra was more dilute, the equivalence zone was reached and

at the highest concentration of antigen, the fall in the amount of antibody precipitated suggests that excess antigen was present, and is consistent with the form of the classical precipitin curve.

TABLE 3

Series C and D

Tube No.	Concentration of antigen added (%)	Dilution of ^{131}I-serum Tra added.	Precipitate Counts per 100 secs.*	
			Series C.	Series D.
1.	100	1 in 64	4140	3576
2.	50	" " "	4470	4125
3.	33	" " "	3411	3581
4.	20	" " "	2298	2395
5.	14	" " "	1669	1771
6.	10	" " "	779	1270
7.	5	" " "	297	612
8.	0	" " "	0	0
9.	20	Normal serum 1 in 64	-	-

*Background (300 counts per 100 secs) and the radioactive count in Tube 8 (11% counts per 100 secs in series C, 1251 counts per 100 secs in series D) have been subtracted from these figures. The counts for tubes 9 were less than those for tubes 8 and the subtraction thus gives negative figures.

4. The use of concentrated antigen solution and ^{131}I-labelled serum Tra. In order to demonstrate more convincingly the inhibition of precipitation in the zone of antigen excess, the quantitative precipitin test with serum Tra labelled with ^{131}I was repeated using a more potent antigen. This antigen was prepared by extracting a thyrotoxic thyroid gland, concentrating a portion of this extract by freeze-drying, and redissolving the freeze-dried residue in a volume of the remaining extract equal to the original volume of the freeze-dried portion. Before use as antigen, this extract was centrifuged at 34,000 g. for one hour. A satisfactory precipitate was observed when this concentrated antigen was tested in an agar-gel diffusion test with serum Tra diluted 1 in 32.

Series K and L. 4 ml. of the concentrated antigenic extract was mixed with 1 ml. of serum Tra used at a dilution of 1 in 27. The precipitates formed were treated in the same manner as before and the results are shown in Table 4 (p. 41) and given graphically in Fig. 8. As with the previous graphs for series A to D, no inhibition of precipitation is shown in the zone of antibody excess and in this respect the curves resemble the classical rabbit type curve described by Heidelberger and Kendall. In the zone of antigen excess, however, there is a very gradual decrease in the amount of precipitate produced.

TABLE 4

Series K and L

Tube No.	Concentration of antigen added (%)	Dilution of ^{131}I-serum Tra added	Precipitate Counts per 100 secs.*	
			Series K.	Series L.
1.	100	1 in 27	3668	3193
2.	88	" " "	3705	-
3.	70	" " "	3484	4219
4.	60	" " "	4431	3813
5.	50	" " "	4460	4050
6.	40	" " "	4971	4761
7.	30	" " "	6073	5609
8.	20	" " "	5309	4622
9.	10	" " "	3563	3606
10.	0	" " "	0	0
M5 and N5	50	Normal serum 1 in 27	M -	N -

* These results have been corrected for background and non-specific precipitation as in the previous experiments.

The supernates from series K were tested for antigen and antibody excess in Ouchterlony agar-gel diffusion plates. The supernates of tubes K1 to K 8 were tested for antigen excess with serum Tra diluted 1 in 32. Bands of precipitate were obtained in agar-gel diffusion tests between serum Tra and the supernates of K1 to K5, thus indicating the presence of free antigen in these supernates. Supernates K7 to K10 were tested in the same manner for antibody excess with thyrotoxic thyroid extract as antigen. Free serum Tra appeared to be present only in the supernate of K10, in which the serum was mixed with saline solution instead of antigen. However, the dilution of serum in the original mixture, and therefore in the supernates, was much higher than the optimal dilution (1 in 32) for agar-gel precipitin tests; moreover, the precipitin in serum Tra was not titrated after labelling with ^{131}I and may have been weakened by this procedure.

Conclusion. The quantitative precipitin curve, produced between serum Tra and dilutions of a thyroid extract antigen, is essentially of the classical ('rabbit') type described by Heidelberger and Kendall, in which there is no inhibition of precipitation in antibody excess. The unusually high protein content of the mixtures may have been responsible for the atypical appearance of the precipitin curve in the region of antigen excess.

Fractionation of tissues.

In an attempt to elucidate the nature of the antigen reacting with serum Tra, fractionation of various tissues was performed to isolate the cellular constituents, i.e. nuclei, mitochondria, microsomes and soluble protein. Two methods of fractionating the tissues were employed. The first was the method of differential centrifugation in 0.25 M sucrose, described by Hogeboom and Schneider (1955). The second method made use of a 1% solution of citric acid in order to provide nuclei free from contamination with cytoplasmic particles or soluble protein (Mirsky and Pollister, 1946, Dounce, 1955).

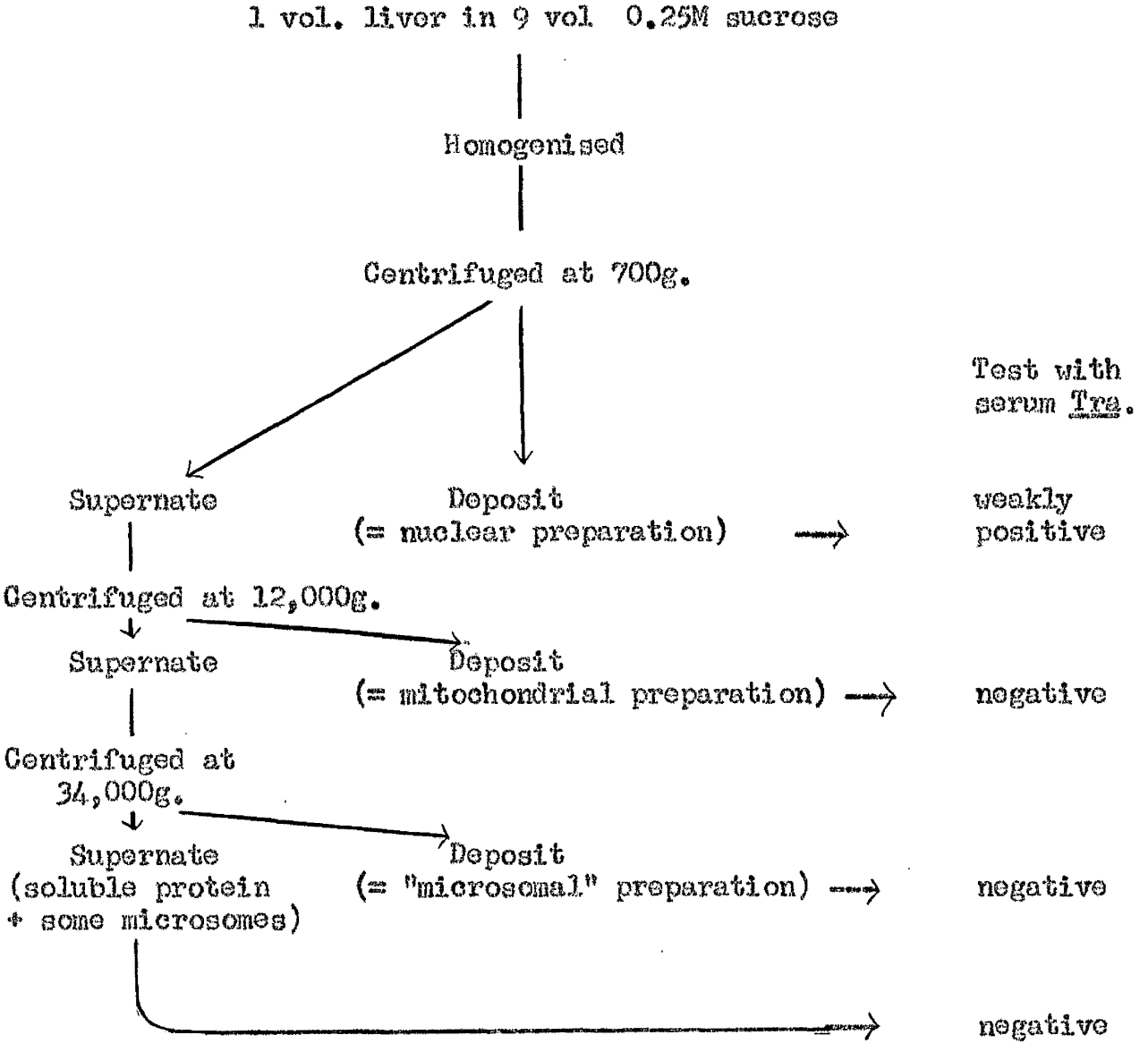
Fractionation in 0.25M sucrose.

This technique has been used mainly to fractionate liver tissue. Three specimens of liver and one of spleen were obtained shortly after death. A temperature of 0-4°C. was maintained throughout the procedure. (Table 5, p.44).

A 10% homogenate of the tissue was made in 0.25M sucrose in a mechanical "Teflon" piston-type homogeniser. This homogenate was centrifuged at 700 g. for 15 minutes. The deposit obtained after centrifugation, examined microscopically, was observed to be composed principally of nuclei and a few intact cells. The supernate, almost free of nuclei, was centrifuged at 12,000 g. for 20 minutes. The deposit at this stage, when examined microscopically was found to consist principally

TABLE 5

Fractionation in sucrose



of mitochondria. Further centrifugation at 34,000 g. for 1 hour gave a deposit composed of very small particles ('microsomes') and a few mitochondria. The deposits obtained at each stage of fractionation were washed once by suspending in 0.9% saline solution, sedimenting again by centrifugation, and resuspending in an equal volume of saline solution.

The fractions were tested immediately without any further treatment and again after freezing and thawing 3 times in order to disrupt the subcellular particles. When tested with 1 in 32 serum Tra in agar-gel diffusion tests, the deposits consisting of nuclei were either negative or reacted to give a faint precipitate; the deposits containing mitochondria and microsomes were invariably negative. The supernatant fluid (soluble protein) of this fractionation was negative with serum Tra. Since this supernate was produced by mixing one volume of tissue with 9 volumes of 0.25M sucrose, it was not unexpected that this fraction failed to react in an Ouchterlony test with serum Tra (see p. 21); the supernate of a 50% crude tissue extract in 0.25M sucrose, centrifuged at 34,000 for one hour was always positive; it is reasonable therefore to assume that the supernate of the 10% tissue extract was too dilute to react with serum Tra.

Citric acid preparation of nuclei.

Microscopic examination of the nuclear preparation obtained in sucrose showed it to contain some whole cells and mitochondria.

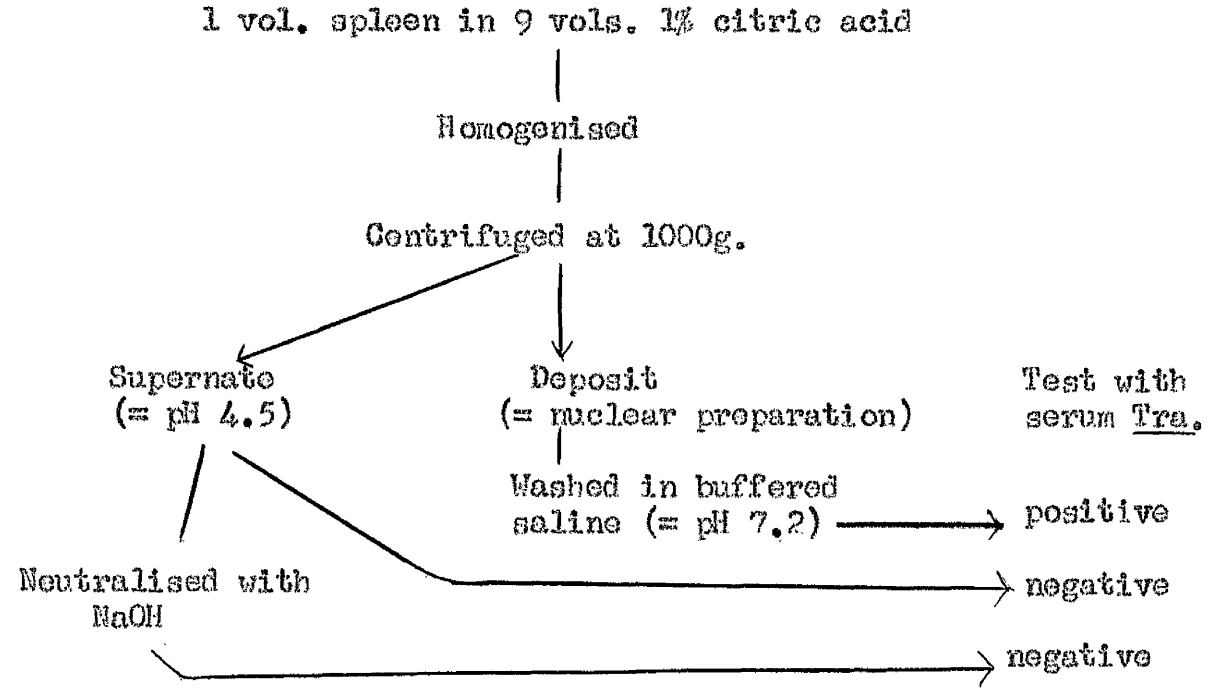
Accordingly, the citric acid method of fractionation was employed to obtain nuclei free from contamination with cytoplasmic constituents.

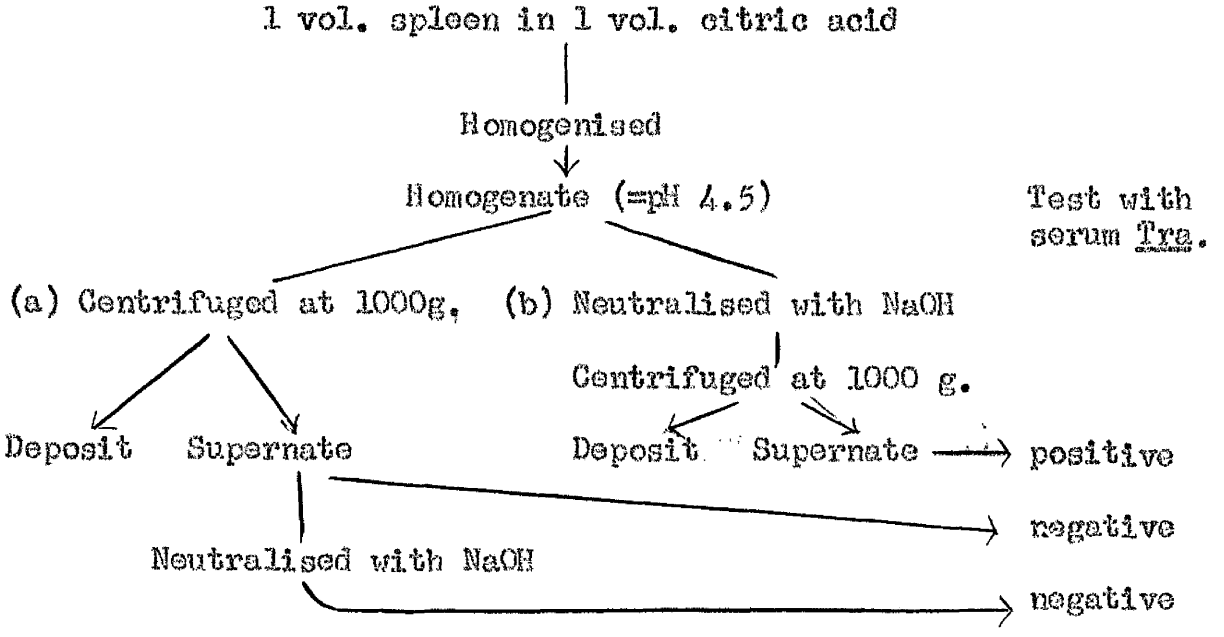
Four specimens of human spleen were extracted by the citric acid method, the temperature being maintained at 0-4°C. throughout this procedure. (Table 6, p. 47). One volume of sieved splenic tissue and nine volumes of 1% citric acid were homogenised and centrifuged at 1000 g. for 15 minutes to deposit the nuclei. When the supernate from this extraction was tested, without further treatment, in an Ouchterlony agar-gel diffusion plate with serum Tra, used at dilutions of 1 in 8 and 1 in 32, no precipitate was obtained. The supernate of a 50% extract of the same spleen in saline, however, gave a positive test with serum Tra. The nuclear deposit of the citric acid homogenate was washed once with an equal volume of buffered saline at pH7.2 and resuspended in buffered saline. This neutralised deposit gave precipitates with both dilutions of serum Tra. The supernate obtained after citric acid homogenisation (pH 4.5) was adjusted to pH 7.2 with dilute sodium hydroxide solution and retested with serum Tra in an agar-gel diffusion plate. This neutralised supernate again failed to give a precipitate with serum Tra.

Sieved splenic tissue was homogenised in an equal volume of 1% citric acid and the homogenate (pH 4.5) was divided into two portions. (a) The first portion of the homogenate was centrifuged at 1000g. for

TABLE 6

Fractionation in citric acid





15 minutes to deposit the nuclei and the supernate tested both before and after treatment with dilute sodium hydroxide solution to adjust the pH to 7.2. Both the untreated and the neutralized supernate failed to give a precipitate with serum Tra. (b) The second portion of homogenate (uncentrifuged) was adjusted to pH 7.2 with dilute sodium hydroxide solution and then centrifuged at 1000 g. for 15 minutes. The supernate of this neutralised homogenate, tested with serum Tra, reacted to give a precipitate in an agar-gel diffusion test.

Acidification of a 50% saline extract of spleen.

The effect of acidification to pH4.5 of a 50% extract of spleen in saline solution was examined. The extract was centrifuged at 17,000 g. for 15 minutes at 4°C. and the supernate adjusted to pH 4.5 by the addition of dilute hydrochloric acid. When this acidified extract was centrifuged at 12,000 g. for 15 minutes, a large deposit was obtained. The supernate was removed, neutralised, and tested in an agar-gel diffusion test; no precipitate was obtained with 1 in 32 serum Tra. The deposit from this acidified extract was suspended in saline solution and adjusted to pH 7.2. This neutralised suspension was positive with serum Tra in an agar-gel diffusion test. The supernate, obtained after centrifugation of the neutralised suspension, was also tested and shown to be positive.

Conclusion. The tissue extract antigen reacting with serum Tra appears

to be precipitated at pH4.5 and redissolves when the pH is adjusted to 7.2.

Absorption of serum Tra with fractions of rabbit liver.

Differential centrifugation was performed on a homogenate of rabbit liver in 0.25 M sucrose, a temperature of 0-4°C. being maintained throughout the procedure. The technique was as already described for human liver and spleen.

The deposits obtained after each centrifugation were washed separately in 0.25 M sucrose and examined microscopically. The first deposit ('nuclear') was shown to consist principally of nuclei but contained also some unbroken cells, and mitochondria; the second consisted mainly of mitochondria and the third contained many very small particles ("microsomes") with few mitochondria.

Serum Tra, diluted 1 in 15, was mixed with equal volumes of the 3 deposits. In the final supernate, serum Tra was added to a final dilution of 1 in 16. These mixtures were kept at 4°C. overnight and centrifuged on the following day at 24,000g. Doubling dilutions of the supernates of these mixtures were tested against antigens consisting of a 50% extract of whole rabbit liver in 0.25 M. sucrose and a 50% saline extract of a thyrotoxic thyroid gland extract. A control consisting of doubling dilutions of an untreated 1 in 16 dilution of serum Tra was also tested at the same time. Only the final supernate (soluble protein)

caused a marked reduction in the titre of serum Tra, the reduction in titre being the same for both antigens.

Conclusion. Fractionation in 0.25M sucrose of the tissues obtained post-mortem was not successful in giving complete separation of the cellular constituents, but the results suggested that the antigen for serum Tra was absent from mitochondria and microsomes. The antigen was always present in the supernate of 50% extracts in 0.25M sucrose, and occasional weak reactions were observed with nuclear preparations. The results of citric acid treatment, which it was hoped would demonstrate the presence of absence of antigen in the cell nuclei, suggested that the antigen was present in the nuclei; however, when the homogenate was neutralised before centrifugation, the antigen appeared in the supernate. The antigen thus appears to be insoluble in 1% citric acid, and its presence in some citric acid preparations of 'nuclei' does not indicate its site within the cell. It may, indeed, be a nuclear constituent extractable by saline or sucrose, but not by citric acid. On the other hand, it is equally possible that the antigen is a cytoplasmic constituent and that it is precipitated and on centrifugation is deposited with the nuclei, in citric acid homogenates.

These inconclusive results are merely an illustration of the uncertainty of the origin of any cellular constituent detected in the supernatant fluid of tissue homogenates.

Complement fixation tests with serum Tra.

In order to determine whether the antibody of serum Tra could be detected by a technique other than the precipitin reaction, complement fixation tests were performed, using extracts of human tissues as antigens. The technique used is described on p. 149.

Serum Tra was tested in doubling dilutions with extracts of fresh post-mortem tissues of thyroid, adrenal, liver, kidney, and salivary gland and with surgically-removed thyrotoxic thyroid gland extracts. Complement fixation titres of serum Tra were high with all tissue extracts tested, generally being of the order of 1 in 512 or higher. "Chessboard" titrations in which each of a series of doubling dilutions of serum Tra was tested with a series of doubling dilutions of tissue extracts, were performed, using thyrotoxic thyroid and parotid gland extracts, by the method of Donnelley (1951) which employs only 2 drop volumes of reagents. A stepwise decrease in titre occurred as the concentration of tissue extract diminished. Table 7 (p. 53) illustrates the results obtained with an antigen extract of thyrotoxic thyroid gland.

Summary.

A precipitating factor, subsequently shown to be an antibody, was detected in the serum of a patient Tra with Sjogren's syndrome. This antibody (anti-SjT) reacted to form a precipitate with extracts of

various human tissues from all individuals tested and with extracts of lymphatic leukaemic leucocytes.

Attempts to determine the intracellular site of antigen SjT were unsuccessful by the methods of tissue fractionation available. Antigen SjT was unaffected by treatment with deoxyribonuclease and ribonuclease and was destroyed by treatment with trypsin and chymotrypsin.

TABLE 7

"Chessboard" titration between serum Tra and thyrotoxic thyroid extract

Dilutions of thyroid extract	Dilutions of serum Tra								
	4	8	16	32	64	128	256	512	1024
1	++	++	++	++	++	++	++	++	-
2	++	++	++	++	++	++	++	++	-
4	++	++	++	++	++	++	+	+	-
8	++	++	++	++	+	-	-	-	-
16	++	++	+	-	-	-	-	-	-
32	+	-	-	-	-	-	-	-	-

++= no lysis; + = partial lysis; - = complete lysis

Further tests for precipitating antibodies to human tissue extracts in the serum of 48 patients with Sjögren's syndrome

In view of the results obtained with serum Tra, serum was obtained from a further 46 patients with Sjögren's syndrome. Diagnosis was made by the clinicians supplying the serum and was based on the patient's case history, clinical observations, and supported by positive Schirmer and rose-bengal tests.

In a study of Mikulicz's disease, Morgan and Castleman (1953) showed that the pathological changes in the salivary glands were closely similar to those found in the lacrimal glands in Sjögren's syndrome and suggested that since the two conditions bore such close pathological and clinical resemblances, they might be anatomical variants of the same disease. Morgan (1954) reinvestigated 18 patients with Mikulicz's disease and concluded that the pathological lesions occurring in the salivary glands were identical with those found in Sjögren's syndrome; clinical features which occur in Sjögren's syndrome, such as rheumatoid arthritis, keratoconjunctivitis sicca, and xerostomia, were also observed in many of the patients with Mikulicz's disease. From this and other studies (e.g. Shearn, 1961), it is apparent that the two conditions cannot readily be separated and in the present study histologically confirmed cases of Mikulicz's disease have been included under the heading of Sjögren's syndrome.

For convenience, sera obtained from patients with either Mikulicz's disease or Sjögren's syndrome, will be referred to as "Sjögren sera."

A total of 48 Sjögren sera, including the 2 sera already tested (p. 16), were examined for precipitating antibodies to human tissue extracts by the agar-gel diffusion technique. 15 sera, initially tested both undiluted and diluted 1 in 8, were found to contain precipitating factors reacting with thyrotoxic thyroid extract. The precipitates produced were very similar in appearance to that given by serum Tra, although some were very slightly concave towards the serum well. Certain sera gave two bands of precipitate, and one (serum Ser) gave three well-separated bands of precipitate when tested undiluted (Fig. 9); at a dilution of 1 in 8, two bands of precipitate persisted with some of these sera; on testing in serial dilutions, one band of precipitate generally disappeared before the other.

All positive sera were titrated, using twofold dilutions of serum, with an extract of thyrotoxic thyroid. Precipitating titres ranged from 1 in 1 to 1 in 512; 5 sera had precipitating titres greater than 1 in 8 (see Table 9, p. 68). In 5 sera the precipitating factors were strong enough to allow of their comparison with each other and with that in serum Tra by means of direct tests for identity and antigen annulment tests.

Specificity of the precipitin reaction between Sjögren sera and human tissue extracts.

In order to determine whether this precipitin reaction was specific for Sjögren sera, 200 specimens of serum obtained from surgical, medical and gynaecological cases were tested for precipitating factors to tissue extracts. Two of these sera were found to give precipitates; one of these reacted only when undiluted and was from a patient with aplastic anaemia who had no history or symptoms of Sjögren's syndrome or of any other connective tissue disease and who had had repeated blood transfusions; the other was from a patient with a parotid swelling which was later diagnosed by biopsy as Mikulicz's disease; this serum reacted at a dilution of 1 in 8 but insufficient was available for titration or other immunological investigations and the patient did not return to hospital.

Serum was also obtained from 33 patients with other salivary and eye conditions, including parotitis, submandibular duct calculus, iritis, orbital granuloma, etc. Only one serum reacted weakly when undiluted and this was from a patient with sialectasis, believed to be due to bacterial infection; no histology was available from this patient, therefore this diagnosis is uncertain.

Comparison of precipitating factors in Sjögren sera.

For the reasons given on p. 23 an extract of thyrotoxic thyroid gland was used for these tests. Tests with extracts of other human

tissues are described on pp. 19-20, 62-64. Some thyrotoxic thyroid extracts were found to be more suitable than others for comparison of serum precipitating factors.

1. Direct tests for identity of precipitating factors in Sjögren sera. Precipitating sera from patients with Sjögren's syndrome were tested for reactions of identity, partial identity and non-identity in agar-gel diffusion tests. The limitations of this method have already been referred to on p. 22. The appearances of such tests are illustrated in Fig. 10 which shows reactions of identity and of non-identity.

When serum Tra (containing antibody designated anti-SjT) was compared with other precipitating sera, it was found that serum from a patient Don (precipitating titre 1 in 64) gave a precipitate which crossed that formed by serum Tra, indicating non-identity of the precipitating factors in these 2 sera. The precipitating factor in serum Don was therefore designated anti-SjD (evidence for its antibody nature is given below). Another serum from a patient Ral gave 2 well-separated bands of precipitate, one of which gave a reaction of identity with serum Tra, indicated by fusion of the precipitates; the second band of precipitate formed by serum Ral crossed that formed by serum Tra and fused completely with the precipitate formed by serum Don. It was therefore concluded that serum Ral contained both anti-SjT and anti-SjD. Another serum was found by

this method to contain both anti-SjT and anti-SjD; this serum (Plu) usually gave only one band of precipitate although 2 bands were seen in some tests. It thus appears that this latter serum Plu contains both factors and that the precipitates are usually superimposed on one another to give a single line. The occasional separation of the precipitate formed by serum Plu into 2 lines is probably dependent upon the proportions of the factors SjT and SjD in the tissue extract used in the test. A third antibody, not detected in the serum from any other patient with Sjögren's syndrome, was found to occur in serum Ser, which also contained both anti-SjT and anti-SjD. This serum gave 3 bands of precipitate when tested undiluted. The precipitating factors in the other 10 sera were too weak for identification.

2. Annulment of antigen by precipitating Sjögren sera. A description of the method of comparing the precipitating factors in sera by annulment of antigen in human tissue extracts is given on p. 146. The results obtained by this technique confirmed the conclusion drawn from the direct identity tests that there are 2 distinct types of precipitating factors among the positive Sjögren sera. The results of these antigen annulment tests are summarised in Table 8. (p. 59).

TABLE 8

Treatment of tissue extract with precipitating Sjögren sera

Tested with serum	Thyroid extract treated with serum of							
	Tra	Don	Tra+Don	Ral	Plu	Scr	Normal serum	Identity of precipitating antibodies
Tra	-	+	-	-	-	-	+	anti-SjT
Don	+	-	-	-	-	-	+	anti-SjD
Ral	+	+	-	-	-	-	+	(anti-SjT (anti-SjD
Plu	+	+	-	-	-	-	+	(anti-SjT (anti-SjD
Scr	+	+	+	+	+	-	+	(anti-SjT (anti-SjD (anti- ? *
Normal serum	-	-	-	-	-	-	-	

* As reported on p.58 this serum contains a third, unidentified precipitating factor.

Precipitating antibodies to thyroglobulin in Sjögren sera.

Since thyroid extract has generally been used for testing these sera in agar-gel diffusion plates, it might be expected that any serum containing precipitating antibody to thyroglobulin would give a positive result. However, the thyroid extract used as antigen in the detection of serum precipitating factor to tissue extracts is much more concentrated than the optimum dilution of thyroid extract used in the detection of antithyroglobulin (Anderson, Buchanan, Goudie and Gray, 1962). Antithyroglobulin precipitates appearing in these tests are therefore very close to the serum well and are observed to migrate even nearer to the serum well when the plates are examined at daily intervals.

Three Sjögren sera, tested using thyrotoxic thyroid extract as antigen, gave precipitates which appeared likely to be due to antithyroglobulin. These sera were therefore tested with a solution of 'purified' thyroglobulin * (Derrien, Michel and Roche, 1948) at a concentration of 5 mg./ml. as antigen. All 3 formed a precipitate with this preparation. The presence of precipitating factors other than antithyroglobulin was confirmed in 2 of these 3 sera using extracts of human tissues other than thyroid. These latter precipitating factors gave weak reactions and could not be identified as either anti-SjT or anti-SjD. None of the other 45 Sjögren sera reacted to form a

*Dr. I.M. Roitt of the Middlesex Hospital, London, kindly provided a supply of purified human thyroglobulin.

precipitate with purified thyroglobulin. The highly sensitive tanned red cell agglutination test for antibody to thyroglobulin was also performed on 26 Sjögren sera, and the results are reported and discussed on pp. 117-125.

The precipitating factor in serum Don (anti-SjD).

Appearance of precipitate.

The precipitate formed by serum Don in agar-gel diffusion tests is generally slightly concave towards the serum well (Fig. 11) unlike that formed by serum Tra which is straight (Fig. 1). The precipitate generally appeared in 24 to 48 hours.

Immunoelectrophoresis.

Immunoelectrophoresis of serum Don was carried out as described on p. 147. This showed that the precipitating factor anti-SjD had the electrophoretic mobility and pattern of gamma-globulin.

Ultracentrifugation.

Fractions of serum Don prepared by Dr. Charlwood were tested in agar-gel diffusion plates. The precipitating factor in serum Don was present only in the 3 fractions which were found by Dr. Charlwood to be those containing 7S gamma-globulin.

From the results obtained by immunoelectrophoresis and ultracentrifugation it is concluded that the precipitating factor in serum Don is an antibody.

Incidence of antigen SjD in human tissue extracts.

The incidence of the precipitating antigenic factor in human

tissue extracts reacting with serum Don in agar-gel diffusion tests appeared at first to correspond rather closely to that of serum Tra. Antigen SjD was demonstrated in extracts of thyroid, adrenal, liver, kidney, spleen, testis, and salivary glands, and in extracts of lymphatic leukaemic leucocytes obtained from peripheral blood. In order to determine whether it was present in the tissues of all individuals, a series of 90 thyroid extracts, including both surgically-removed and post-mortem material which had been stored at -20°C. for various periods up to 2 years, were tested with serum Don used at its optimal dilution of 1 in 16. Approximately half of these extracts reacted to form precipitates with serum Don. A series of extracts of thyroid and either spleen or submandibular gland obtained post-mortem from 40 individuals were made and tested with serum Don immediately after extraction; 55 of the 80 extracts reacted with serum Don. Reactions were observed with both extracts from 20 individuals, and with either one, but not both, extracts from 15 individuals. Thus the antigen was detectable in the tissues of 35 of the 40 individuals tested. Since the positive tests with serum Don indicated that the antigen was present in very small amounts in some of the tissue extracts, the extracts from the 5 individuals giving negative results were concentrated by freeze-drying the extracts and redissolving the residue in water to half the original volume. On testing these concentrated extracts, positive results were obtained with the tissues of 3 of the 5 individuals.

Of the 2 individuals whose tissue extracts did not react with serum Don, one was a woman who had died of progressive systemic sclerosis. Her tissue extracts were observed to react to form a precipitate with the tissue extracts of other individuals. As shown later, patients with progressive systemic sclerosis frequently have precipitating antibodies, including anti-SjD, in their serum. Unfortunately, serum was not available from this patient, but the failure of her tissue extracts to react with serum Don, and their reaction with other tissue extracts, strongly suggests that this patient had anti-SjD in her serum and that this antibody, present in the blood within the tissues, had completely annulled the SjD factor in the tissue homogenates. The other individual whose tissue failed to react with serum Don died after gastrectomy for carcinoma of the stomach. Before operation, she had a reaction after being transfused for a second time with 2 to 3 pints of blood. This patient had a history of dry eyes and mouth; she had what had been regarded as a nodular goitre although 7 years previously she had been shown to have a low basal metabolic rate (-40%). It is thus possible that this patient was a case of Sjogren's syndrome, associated as it commonly is, with chronic thyroiditis. Post-mortem examination of the thyroid was not performed in this case.

Incidence of antigen SjD in animal tissues.

Liver, kidney and spleen extracts were prepared from organs obtained from rabbits, guinea pigs, rats, and mice, and were tested immediately after extraction. Serum Don was found to be positive only with extracts of guinea pig organs.

Effect of heat on anti-SjD and on tissue extracts containing antigen SjD

Serum Don (containing anti-SjD) was heated for 2 hours at 55°C. Heated and unheated serum at dilutions of 1 in 8 and 1 in 64 was then tested for anti-SjD and equally good reactions were obtained with the heated as with the unheated serum dilutions.

Specimens of a thyrotoxic thyroid extract were heated at temperatures of 37°C., 45°C., 55°C., and 65°C. for 30 minutes. The results of precipitation tests, using serum Don diluted 1 in 16, showed that antigen SjD was not destroyed after heating at temperatures up to 55°C. After heating at 65°C., however, antigen SjD was not detected in the extract.

The effect of enzymes on antigen SjD

Experiments involving treatment of tissue extracts with crystalline trypsin, chymotrypsin, deoxyribonuclease and ribonuclease have already been described on pp. 27 - 28 where the treated extracts were tested with serum Tra. Enzyme-treated extracts were also tested in

Ouchterlony tests with serum Don. Tissue extracts treated with trypsin and chymotrypsin gave a slightly weaker reaction with serum Don than with the untreated control extract which had been incubated at 37°C. for the same length of time as the enzyme-treated extracts. Deoxyribonuclease and ribonuclease were without effect on antigen SjD.

Summary.

Antigen SjD resembles antigen SjT in that it occurs in a wide variety of human tissues from all individuals tested and in lymphatic leukaemic leucocytes; it is unaffected by treatment with deoxyribonuclease and ribonuclease. Unlike antigen SjT, antigen SjD deteriorates when stored at -20°C. (p. 63). On the other hand, antigen SjD resists heating to a higher temperature than antigen SjT and is more resistant to treatment with the enzymes trypsin and chymotrypsin.

Immunoelectrophoresis of the sera of other patients with Sjögren's syndrome.

Immunoelectrophoresis was also performed on serum Ral and serum Plu and the precipitating factor in both these sera shown to have the electrophoretic mobility and pattern of gamma-globulin.

Complement fixation tests with serum from patients with Sjögren's syndrome.

Complement fixation tests were performed on Sjögren sera by the method described on p. 149 using extracts of normal human thyroid and kidney as antigens. Sera containing anti-SjT usually reacted to fix complement with both antigens. Sera containing anti-SjD varied in their ability to fix complement. Complement-fixing sera usually reacted to the same titre with both kidney and thyroid extracts.

TABLE 9

Complement fixation reactions with precipitating Sjögren sera

Serum	Complement-fixing titre with extracts of		Precipitating titre	Identity of precipitating antibody
	Thyroid	Kidney		
Tra	512	1024	256	anti-SjT
Don	4	4	64	anti-SjD
Ral	1024	1024	64	(anti-SjT (anti-SjD
Plu	256	256	64	(anti-SjT (anti-SjD
Kie	4	4	4	anti-SjD*
Har	4	4	1	not identified
Wal	4	4	4	"
War	4	16	4	"
Ser	64	256	512	(anti-SjT (anti-SjD (anti- ?

*Identified only by direct tests for identity.

Discussion

Sjögren's syndrome.

Heaton (1959) has drawn attention to the clinical and pathological similarities occurring between Sjögren's syndrome and systemic lupus erythematosus, and between Sjögren's syndrome and Hashimoto's thyroiditis. As result of these observations, he suggested that an auto-immune phenomenon, for which strong evidence exists in systemic lupus erythematosus and Hashimoto's thyroiditis, might also be concerned in Sjögren's syndrome. Moreover, Jones (1958) had described the occurrence of precipitating factors reacting with lacrimal and salivary tissue in the serum of patients with Sjögren's syndrome. The present investigation is an attempt to determine whether precipitating auto-antibodies are detectable in Sjögren sera, and if so, whether they react specifically with salivary or lacrimal tissue constituents.

Of 48 sera from patients with Sjögren's syndrome, 15 were found to contain precipitating factors which reacted not only with human salivary tissue extracts, but with a wide range of human and certain animal tissue extracts. These reactions were demonstrated in Ouchterlony agar-gel diffusion tests in which the wells containing the serum and tissue extracts were larger, and more closely spaced than is customary in such tests. The precipitating titres of tissue extracts were low and dilution of the extract resulted in dimimution in the amount of

precipitate. In contrast, the serum from some patients was of high titre, 2 sera reacting at dilutions up to 1 in 256. These findings disagreed with those described by Jones in that he found that the serum reacted only when undiluted and his extracts were appreciably more dilute than those used in this investigation.

Comparison of the precipitating factors was possible with sera of high titre. These factors were shown to be of two distinct types, termed anti-SjT and anti-SjD; one serum (Scr) contained a third factor, in addition to the other two.

The antibody nature of anti-SjT and anti-SjD was established by showing that they were 7S gamma-globulins and exhibited distinct specificity; tissue extracts annulled by treatment with one antibody remained capable of reacting with the other. Furthermore, by labelling serum Tra (anti-SjT) with radioactive iodine (^{131}I), a quantitative precipitin curve, of classical form, was demonstrated by adding increasing concentrations of a tissue extract to constant amounts of serum Tra.

The occurrence of the corresponding antigen in the tissues of all individuals tested, suggests that anti-SjT and anti-SjD are autoantibodies. Their auto-immune nature was confirmed (see p. 107) when tissue extract from an individual with rheumatoid arthritis, whose serum contained both antibodies, was found to react with her own serum, with serum Tra (anti-SjT) and with serum Don. (anti-SjD).

Anti-SjT and anti-SjD were shown to react with extracts of all highly cellular tissues tested. The intracellular nature of the corresponding antigens was established by their detection in extracts of lymphatic leukaemic leucocytes. Attempts to establish the intracellular site of antigen SjT by tissue fractionation demonstrated its presence in 'cell sap' (soluble protein) and inconstantly in nuclear extracts; these results do not reveal whether the antigen is of nuclear or cytoplasmic origin, for in any cell fractionation there is the possibility that material will diffuse out of one of the particulate cellular constituents and appear in the 'cell sap' while preparations of subcellular particles may contain precipitated cytoplasmic material. Consequently this method is of limited value.

Antigen SjT was shown to be more susceptible to heat and the action of proteolytic enzymes than antigen SjD and may thus be of protein nature. The precipitate between serum Don and antigen SjD was slightly concave towards the serum well, suggesting an antigen of lower molecular weight than 7S gamma-globulin (Korngold and Van Leeuwen, 1957). The resistance of antigen SjD to the effects of heat and enzyme action suggests that it is probably not a protein.

Jones considered that the precipitating factor in Sjögren sera was likely to be specific for lacrimal and salivary tissue extracts, but nevertheless he reported that it reacted also with certain human kidney

extracts. Anti-SjT and anti-SjD are certainly not organ-specific. Their reactivity with a wide range of human and certain animal tissue extracts is in sharp contrast to the behaviour of the antibodies present in the serum in the majority of cases of Hashimoto's thyroiditis which react specifically with thyroid tissue constituents.

I am not aware of any other reports describing the occurrence of precipitating autoantibodies in Sjögren's syndrome although antibodies have been demonstrated by the technique of complement fixation and the fluorescent antibody technique for anti-nuclear factors.

Complement fixation reactions between Sjögren sera and human and animal liver and kidney antigens were reported by Deicher, Holman and Kunkel (1960). Bloch, Wohl, Ship, Oglesby and Bunim (1960) reported complement-fixation occurring between serum from patients with Sjögren's syndrome and extracts of human submandibular gland, liver, kidney, and striated muscle. Such non-organ-specific complement fixation reactions were also described by Gajdusek (1958) as the "auto-immune" complement fixation (A.I.C.F.) reaction, which he reported as occurring in high titre in the sera of some patients with systemic lupus erythematosus, primary biliary cirrhosis and macroglobulinaemia. Heaton (1962) has reported a higher incidence of A.I.C.F. reactions, using human thyroid and liver or kidney antigens, in a series of patients with Sjögren's syndrome than in a series of patients with systemic lupus erythematosus.

In the present investigation, complement-fixation tests were performed using extracts of human thyroid and kidney tissues as antigens. A few sera contained factors which reacted to fix complement with both tissues, the titre being approximately the same for each tissue extract. Comparison of the complement-fixing titres with the precipitating titres showed that serum containing anti-SjT generally reacted to fix complement and the titres of precipitating and complement-fixing antibodies were of the same order in each serum. Sera which contained anti-SjD varied in their ability to fix complement, however, and most, but not all, non-precipitating sera failed to fix complement.

The fluorescent technique for the detection of intracellular antigens was performed by Dr. J.S. Beck on 44 of the sera from patients with Sjogren's syndrome. The tests were performed using unfixed sections of rat and frog livers. The sections were treated first of all with the patient's serum, washed to remove unattached gamma-globulin, treated with fluorescein-conjugated rabbit anti-human gamma-globulin and then washed again to remove unattached serum-conjugate. The presence in the test serum of antibody to the tissue constituents, was thus made visible in ultraviolet light by the attachment of fluorescent serum-conjugate to antibody gamma-globulin. Four distinct patterns of fluorescence were observed in these sections; these were diffuse cytoplasmic, homogeneous nuclear, speckled nuclear and nucleolar fluorescent staining. (Beck,1961)

Positive results occurred both in sera which contained precipitating antibodies and in non-precipitating sera. The fluorescent antibody titres were, in general, higher in sera which contained precipitating antibodies. It was shown that sera containing anti-SjT invariably gave speckled nuclear and diffuse cytoplasmic staining but the titres of neither bore any relationship to the titres of precipitating antibody. Sera containing anti-SjD produced diffuse cytoplasmic and either homogeneous or speckled nuclear fluorescence. The nucleolar pattern of fluorescence was encountered in only 2 sera, one of which contained no detectable precipitating antibody and the other was serum Scr., which contained both anti-SjT and anti-SjD and also a third precipitating factor. Since antigen SjT and antigen SjD have not been demonstrated in rat tissues in precipitin tests, it was important to ascertain whether these patterns of nuclear, nucleolar and cytoplasmic fluorescence were also obtained with human tissue sections. Using a few, selected Sjögren sera and tissue sections of human thyroid and liver, Dr. Beck demonstrated the same patterns of fluorescence that he had found previously with tissue sections of rat and frog livers. The pattern of fluorescent staining obtained with Sjögren sera could not be correlated with the presence of either anti-SjT or anti-SjD. Both precipitating antigens, however, have been shown intracellularly and it is difficult to understand why they cannot be demonstrated by this very sensitive fluorescent antibody

technique. It may be that the antigens concerned in the precipitin reaction are very soluble and diffuse out of the tissue sections during their preparation and processing.

The possible significance of the occurrence of precipitating antibodies in Sjögren's syndrome is discussed further in the general discussion on pp. 126-140.

SECTION II

PRECIPITATING ANTIBODIES TO TISSUE CONSTITUENTS IN THE SERUM OF PATIENTS WITH CONNECTIVE TISSUE DISEASES

In the previous section, precipitating factors, regarded as auto-antibodies to tissue constituents, were demonstrated in the serum of patients with Sjögren's syndrome. In view of the association of Sjögren's syndrome with the connective tissue diseases (Cardell and Gurling, 1954, Heaton, 1959, Bunim, 1961, Shearn, 1960, 1961), it was decided to test serum from patients with connective tissue diseases for antibodies by the same technique. Serum was obtained from patients with systemic lupus erythematosus, discoid lupus erythematosus, progressive systemic sclerosis, dermatomyositis, and rheumatoid arthritis. These sera were tested in agar-gel diffusion plates, with the following results.

Systemic lupus erythematosus

Of 29 cases of systemic lupus erythematosus, 20 were found to have in their serum precipitating factors to human tissue extracts. In 15 of these positive cases, tests were performed on 2 or more specimens of serum obtained at intervals ranging from one month to 2 years; 12 patients had serum specimens which were positive on all occasions and 3 patients gave specimens which were positive only once. The serum factors frequently became weaker or disappeared during steroid therapy; this

change was noticed within one month of the commencement of therapy. The stronger serum precipitating factors detected in this series of tests were compared with each other and with anti-SjT and anti-SjD by direct tests of identity in Ouchterlony plates and by antigen annulment tests as described on p.146. Anti-SjT and anti-SjD were unequivocally identified and in addition 3 other types of serum precipitating factor were demonstrated. The identification of these 3 serum precipitating factors is described on the following pages.

I The precipitating factor in serum Dun

This serum was obtained from a patient (Dun) with untreated systemic lupus erythematosus. Agar-gel diffusion tests performed between this serum and human tissue extracts demonstrated a serum precipitating factor with a titre of 1 in 64. This precipitate was generally visible in 18 to 48 hours. Direct tests for identity and antigen annulment tests were therefore performed on this serum.

Direct tests for identity between serum Dun and a precipitating serum containing anti-SjT and anti-SjD.

Serum Dun tested undiluted gave 2 and sometimes 3 bands of precipitate with thyrotoxic thyroid extracts and, diluted 1 in 8, only one band of precipitate. This serum was therefore compared at both concentrations with serum Tra (anti-SjT), serum Don (anti-SjD) and serum

Plu (anti-SjT + anti-SjD), by the method described on p. 57 using as antigen a thyrotoxic thyroid gland extract. All 3 bands of precipitate produced by undiluted serum Dun crossed the single bands of precipitate produced by the other sera (Fig. 12); at a dilution of 1 in 8 the single band of precipitate of serum Dun crossed those of the other 3 sera. The precipitating factor in serum Dun is therefore not identical with anti-SjT or anti-SjD.

Annulment of antigen by serum Dun.

Three volumes of a thyrotoxic thyroid gland extract were mixed with one volume of serum Dun and tested, as described (p. 146), with sera containing precipitating antibodies anti-SjT and anti-SjD. The treated tissue extract no longer reacted with serum Dun but still gave precipitates with serum Tra (anti-SjT), serum Don (anti-SjD), and serum Plu (anti-SjT + anti-SjD). Thyrotoxic thyroid gland extract, treated in the same way with either serum Tra or serum Don, reacted with serum Dun.

Conclusion.

The results given by these antigen-annulment tests thus confirm those of the direct tests for identity, showing that the precipitating factor in serum Dun is neither anti-SjT nor anti-SjD. Since the patient Dun in whose serum this factor was demonstrated was suffering from systemic lupus erythematosus,

this factor was designated anti-Lup.

The nature of the precipitating factor (anti-Lup)in serum Dun.

Immunoelectrophoresis. Immunoelectrophoresis of serum Dun was performed and the precipitating factor was demonstrated to have the electrophoretic mobility and pattern of gamma-globulin.

Conclusion. The precipitating factor in serum Dun appears to be an antibody.

Ultracentrifugation. Ultracentrifugation of serum Dun was performed by Dr. P.A. Charlwood. None of the resulting fractions, tested in agar-gel diffusion tests, gave a precipitate. The fractions were therefore concentrated to half their original volume and retested, but once more no precipitates were observed with any of these fractions. It is likely that the serum factor was too dilute in these fractions, even after concentration, to form a precipitate,

Incidence of antigen Lup in human tissue extracts.

Tissue extracts prepared from human spleen, thyroid, adrenal, kidney, testis, brain and salivary glands all reacted with serum Dun; since it is unknown whether the 3 bands of precipitate formed with undiluted serum Dun represent 3 antibodies, the serum was used at a dilution of 1 in 8 when only one band of precipitate, probably formed

by a single antigen-antibody reaction, occurs. Serum Dun also reacted with extracts of lymphatic leukaemic leucocytes, whereas no reaction was observed with extracts of myeloid leukaemic leucocytes. Other experiments with extracts of myeloid and lymphatic leukaemic leucocytes are reported on p.83. Some extracts of washed, normal leucocytes also reacted to give a faint band of precipitate with serum Dun, but this was inconstant, some extracts being negative. Extracts of whole blood, platelets, and pooled gamma-globulin did not react with serum Dun. Extracts of cultured human skin fibroblasts from 4 individuals (see p. 20) reacted to form a band of precipitate with serum Dun. The organs obtained post-mortem from 40 individuals and tested with serum Don (p. 63) were also tested, immediately after extraction, with serum Dun. All these extracts reacted to form a precipitate with serum Dun.

In view of the reported occurrence of antibodies to deoxyribonucleoprotein and to deoxyribonucleic acid in the sera of some patients with systemic lupus erythematosus (e.g. by Ceppellini, Polli and Celada, 1957; Deicher, Holman and Kunkel, 1959), serum Dun was tested by the Ouchterlony technique with these compounds. Deoxyribonucleoprotein (DNP) and deoxyribonucleic acid (DNA) were prepared by the method described by Mirsky and Pollister (1946). The crude nucleoprotein at first obtained was found to react with serum Dun, but when purified by reprecipitation and solution, DNP failed to react. Preparations of DNA also failed to

react and neither purified DNP nor DNA annulled the antibody. Histone, prepared by the method of Davison, James, Shooter and Butler (1954), was also negative in Ouchterlony tests and in annulment tests with anti-Lup.

Incidence of antigen Lup in animal tissue extracts.

Extracts of liver, kidney and spleen from rabbits, rats, guinea pigs and mice were prepared from organs taken from freshly killed animals. Serum Dun (diluted 1 in 8) reacted with all these extracts. DNA, prepared commercially from calf thymus nuclei (Nutritional Biochemicals Corporation) was tested at dilutions ranging from 12.5 to 500 ug/ml. with serum Dun both undiluted and diluted 1 in 8, but no precipitation was obtained, although this range of concentrations of DNA was shown to be suitable for precipitation with anti-DNA (see p. 99). This preparation of DNA at 500 ug/ml. was also treated with an equal volume of serum Dun (diluted 1 in 2) in order to test whether the serum antibody could be annulled by this preparation; the reactivity of serum Dun was unaffected by this treatment. Serum Dun was also tested undiluted with a solution containing 25 ug/ml. of commercial DNA which had been subjected to ultrasonic disintegration in an atmosphere of hydrogen - a process which disrupts DNA into smaller fragments, more readily diffusible in agar, without apparently altering its immunological reactivity (Seligmann, 1958). No precipitin reaction was detected between serum Dun and this

preparation. Yeast ribonucleic acid (RNA) was also tested at a concentration of 100 ug/ml. with serum Dun but no reaction was observed.

Conclusion. Anti-Lup is not an antibody reacting with either DNP or DNA. This is supported by the resistance of antigen Lup to treatment with deoxyribonuclease (p. 83).

Effect of heat on anti-Lup and on tissue extracts containing antigen Lup.

Specimens of serum Dun were heated for periods of 30 minutes at 55°C., 65°C., and 75°C. and tested at a dilution of 1 in 8 with a thyrotoxic thyroid gland extract. Heating at 55°C. did not affect the precipitating property of serum Dun but the precipitate produced after heating at 65°C. was rather faint and no precipitate was detected after heating at 75°C.

Specimens of a thyrotoxic thyroid gland extract were heated for 30 minutes at 37°C., 45°C., 55°C., and 65°C. and tested with serum Dun diluted 1 in 8. A strong precipitin reaction was obtained only with the extract heated at 37°C.

Effect of enzymes on antigen Lup.

A thyrotoxic thyroid gland extract, treated with papain at concentrations of 0.1% and 0.2% at 37°C. for 4 hours, was tested in agar-gel diffusion plates with serum Dun diluted 1 in 16. Antigen Lup was weakened by 0.1% papain solution and destroyed by 0.2% papain solution.

In similar experiments, crystalline trypsin, used at concentrations of 0.4%, 0.2%, and 0.1% destroyed antigen Lup in the thyrotoxic thyroid gland extract; chymotrypsin at a concentration of 0.4% also destroyed antigen Lup.

The effect of deoxyribonuclease and ribonuclease on an antigen-Lup was determined by treating a thyrotoxic thyroid gland extract with the enzymes in the same way as for antigen SjT, described on p.27. Both the enzymes were without demonstrable effect on antigen Lup. (Table 10).

In view of the reported occurrence in systemic lupus erythematosus of serum antibodies reacting with degradation products of DNA (Stollar and Levine, 1961), a solution containing 100 ug/ml. of commercial calf-thymus DNA was incubated at 37°C. with deoxyribonuclease for 2 hours and tested with serum Dun diluted 1 in 8. No precipitation was observed.

An extract of lymphatic leukaemic leucocytes, incubated with an equal volume of an extract of myeloid leukaemic leucocytes for 6 hours at 18°C. and tested in an agar-gel diffusion plate with serum Dun, produced a much weaker precipitate than the same extract of lymphatic leukaemic leucocytes diluted with an equal volume of saline solution and incubated for a similar period. A similar result was obtained with an extract of thyrotoxic thyroid extract incubated with an extract of myeloid leukaemic leucocytes. These results suggest that there is in

TABLE 10

Reactions of anti-Lup with enzyme-treated antigenic tissue extracts

Antigen treated with proteolytic enzymes	Enzyme concentration (%)	Reaction with serum Dun (1 in 8)	Antigen treated with	Reaction with serum Dun (1 in 8)
Papain	0.1	weak	DNAase	+
	0.2	-		
Trypsin	0.1	-	RNAase	+
	0.2	-		
	0.4	-	Saline control	+
Chymotrypsin	0.4	-		

extracts of myeloid leucocytes a substance, possibly enzymic, which inhibits the reaction between serum Dun and its corresponding antigen.

Complement fixation tests with serum Dun.

Extracts of fresh post-mortem tissues, including thyroid, adrenal, kidney, and surgically-removed thyrotoxic thyroid gland extract, all fixed complement with serum Dun when tested by the method described on p. 149. The complement-fixing titre of serum Dun was approximately 1 in 16 with all of these extracts. Complement fixation between human tissues and serum from patients with systemic lupus erythematosus has been reported by Gajdusek, (1958), Deicher, Holman and Kunkel (1960), and Hackett, Beech and Forbes (1960). Robbins, Holman, Deicher and Kunkel (1957), Ceppellini, Polli and Celada (1957), Pearson, Craddock and Simmons (1958), and Seligmann (1958), all reported complement fixation between DNA and serum from some patients with systemic lupus erythematosus. Accordingly, complement fixation tests were performed between DNA at a concentration of 10 ug/ml. and serum Dun but were negative.

Conclusion

The antigen reacting with serum Dun appears from these results to be present in the tissues of all humans and of all other mammals tested.

Since this antigen occurs in washed leucocytes, it appears to be an intracellular constituent. It is present in a crude nucleoprotein complex, but not in more highly purified DNP, histone, or in DNA prepared either from human tissue or commercially prepared from animal tissue. The antigen is destroyed by the action of certain proteolytic enzymes. The limited number of complement fixation tests performed suggest that anti-Lup may also be demonstrated by this technique.

Serum precipitating factors in systemic lupus erythematosus

II Serum McL.

Serum from a patient McL was found to precipitate, in agar-gel diffusion tests, with human tissue extracts, up to a dilution of 1 in 512. This patient was believed to be a case of systemic lupus erythematosus although repeated L.E. cell tests were negative. She had a transient arthritis and a haemolytic anaemia, as a result of which she had received a number of blood transfusions. Latterly she developed transfusion reactions after receiving matched blood.

The reaction between serum McL and human tissue extract was more rapid than that occurring with the other serum precipitating factors, being visible within 18 hours. When a thyrotoxic thyroid extract was titrated in doubling dilutions with serum McL, used at its optimal dilution of 1 in 32, the precipitating titre of the extract was 1 in 8; the serum precipitating factors anti-SjT, anti-SjD and anti-Lup, did not generally react with extract diluted more than 1 in 2 or 1 in 4. The precipitate occurring with serum McL was a well-defined band which appeared concave towards the serum well. In this respect it resembled anti-SjD and differed from anti-SjT and anti-Lup which gave straight bands or bands curved towards the antigen well (Fig. 11). Direct tests for identity and tests of antigen annulment were performed on serum McL.

Direct tests for identity between serum McL and the serum precipitating antibodies previously described.

When serum McL was compared with serum Tra (anti-SjT), serum Don (anti-SjD), and serum Dun (anti-Lup) by the method described on p. 57 reactions of non-identity were always observed between serum McL and these 3 sera and also with other sera which had been shown to contain one or more of these 3 antibodies.

Annulment of antigen by serum McL

Nine volumes of a thyrotoxic thyroid extract were treated with one volume of serum McL as described on p.146 and the treated extract was then tested with serum Tra (anti-SjT), serum Don (anti-SjD), serum Plu (anti-SjT + anti-SjD), and serum Dun (anti-Lup); treatment with serum McL did not annul the antigen for any of these antibodies, all of which reacted with the treated extract; serum McL, which reacted with the untreated extract, failed to give a precipitate with the treated extract.

The reactions of non-identity of serum McL with other sera containing known precipitating antibodies and the results of the antigen annulment tests are thus in agreement in showing that serum McL contains a precipitating factor different from those previously described.

Nature of the precipitating factor in serum McL

Immunoelectrophoresis. Immunoelectrophoresis of serum McL, performed as described on p.147 showed that the precipitating factor had the electrophoretic mobility and pattern of gamma-globulin.

Ultracentrifugation. Ultracentrifugation of serum McL was performed by Dr. Charlwood, and the resulting fractions were tested in agar-gel diffusion plates. Bands of precipitate were formed between the tissue extract and those fractions which contained the 7S gamma-globulins.

The results of immunoelectrophoresis, ultracentrifugation and antigen annulment tests provide strong evidence that the precipitating factor in serum McL is an antibody. In the account which follows, it is termed anti-McL.

Incidence of antigen McL in human tissue extracts.

The tissue precipitating antigen for serum McL was shown to be present in extracts prepared from human thyroid, kidney, spleen, salivary gland, heart, testis and brain. In these tests, serum McL was used at its optimal dilution of 1 in 32. It appeared however, that this antigen was either very weak or completely absent from the tissues of certain individuals (Fig. 13). Extracts of organs obtained post-mortem from 40 individuals and tested with serum Don (p.63) were also tested,

immediately after preparation, with serum McL. The tissue extracts from 29 individuals reacted to form a precipitate with serum McL. The tissue extracts from the remaining 11 individuals which gave negative results were freeze-dried, reconstituted in water to half their original volume, and retested. No precipitates developed between serum McL and any of these concentrated extracts, although it was shown that an extract containing antigen McL could be concentrated by this technique of freeze-drying. One of these concentrated extracts, a thyroid extract, reacted to produce a band of precipitate with an extract of spleen known to contain antigen McL, and when compared with serum McL by a direct identity test (p. 22) it was found that the precipitate formed by the concentrated thyroid extract fused completely with that of serum McL. This thyroid extract, originally prepared as an antigen, therefore appeared to contain antibody identical to that found in serum McL. This was confirmed by testing the thyroid extract with 6 tissue extracts, 3 of which contained antigen McL and 3 of which did not contain this antigen, and showing that it reacted only with extracts containing antigen McL. This precipitating antibody was also shown to be present in extracts of spleen, heart, kidney and brain from the same individual. This patient had died 3 days after the excision of a large, malignant rectal polyp and 17 days after receiving 2 pints of matched blood. A specimen of blood removed 10 days after this transfusion could not be shown to contain anti-McL.

The patient had a history of previous abdominal operations for peptic ulcer, and on a previous occasion 9 years before he had been transfused with 4 pints of matched blood.

Human serum and plasma from 6 individuals were examined for antigen McL but all failed to react with serum McL. Antigen McL was detected in some extracts of lymphatic leukaemic leucocytes but not in either of two extracts of myeloid leukaemic leucocytes from different individuals. A reaction between serum McL and one extract of normal leucocytes was not reproducible, and heating the leucocytes of this individual at 56°C. in an attempt to inhibit possible enzymic changes did not provide an extract antigenic for serum McL. The extracts of cultures of skin fibroblasts from 4 individuals, which were previously tested with other precipitating sera (pp. 20, 80), did not react with serum McL.

Deoxyribonucleoprotein, prepared in this department by the method of Mirsky and Pollister (1946) from a spleen which had been shown to contain antigen McL neither reacted with nor annulled the precipitating antibody in serum McL.

Incidence of antigen McL in animal tissue extracts.

Extracts of liver, kidney, and spleen were prepared from freshly killed animals and tested with serum McL diluted 1 in 32. None of these organ extracts, obtained from rabbits, rats, guinea pigs and mice, reacted

to form a precipitate with serum McL. Deoxyribonucleic acid from calf thymus nuclei (Nutritional Biochemicals Corporation) was submitted to ultrasonic disintegration in an atmosphere of hydrogen and tested at a concentration of 25 ug/ml with serum McL used undiluted. No reaction was observed between DNA and serum McL.

Effect of heat on anti-McL and on tissue extracts containing antigen McL.

Specimens of serum McL were heated at temperatures of 55°C., 65°C., and 75°C. for 30 minutes and tested at a dilution of 1 in 32 in agar-gel diffusion plates with a thyrotoxic thyroid extract containing antigen McL. Heating at 55°C. did not affect the precipitin reaction of serum McL; heating at 65°C. diminished the intensity of the precipitate and after heating at 75°C. the serum failed to react to give a precipitate.

By similarly heating portions of a thyrotoxic thyroid extract, its antigenicity for serum McL was found to be unaffected by heating up to 55°C., but was abolished by heating at 65°C. and 75°C.

Effect of enzymes on antigen McL.

The enzyme-treated portions of thyrotoxic thyroid extract, prepared as described on p.27 using the enzymes papain, crystalline trypsin, chymotrypsin, diastase, deoxyribonuclease and ribonuclease respectively, were also tested in agar-gel diffusion plates with serum McL. Extracts

treated with 0.4% chymotrypsin failed to react with serum McL whereas treatment with the other enzymes was without noticeable effect on antigen McL. Thyrotoxic thyroid extract incubated with an equal volume of an extract of myeloid leukaemic leucocytes for 3 hours at 37°C. and at 4°C. overnight still gave a distinct precipitate with serum McL.

Complement fixation with serum McL.

Complement fixation tests were performed by the method described on p.149 using extracts of post-mortem kidney, submandibular gland and thyroid gland. Serum McL failed to fix complement with the extracts of 2 individuals who lacked the tissue precipitating factor reacting with this serum. The extracts of another individual containing antigen McL reacted to fix complement with serum McL at serum dilutions up to 1 in 64.

Conclusion.

Antigen McL is unlike the precipitating factors reacting with anti-SjT, anti-SjD and anti-Lup in that it does not occur in the tissues of all individuals tested and was not shown to be present in any of the animal tissues tested. The shape of the precipitate, concave towards the serum well, and the fact that it appears more quickly than that of the other precipitating factors, suggests that antigen McL is a smaller molecule, more readily diffusible in agar than antigens SjT, SjD or Lup.

It was detected in the tissue of 73% (29 out of 40) individuals. Further discussion on the nature of anti-McL follows the description of its detection in the serum of other individuals (pp. 108, 114).

Serum precipitating factors in systemic lupus erythematosus

III Serum McDou and serum Tan

The serum from a patient McDou with systemic lupus erythematosus precipitated with human tissue extracts in Ouchterlony agar-gel diffusion plates. The precipitating titre of this serum was only 1 in 4 but it nevertheless produced a well-defined band of precipitate in 24 to 48 hours which enabled it to be compared with the other factors by tests of identity and antigen-annulment tests. Another serum from a patient Tan, also with systemic lupus erythematosus, was investigated at the same time as serum McDou. When tested in an agar-gel diffusion plate, serum Tan reacted undiluted to form 2 well-defined bands of precipitate. At a dilution of 1 in 2, serum Tan still reacted with tissue extract but precipitation was less than with the undiluted serum and only one band was formed. It was possible to use serum Tan undiluted for direct tests of identity with other precipitating sera and for antigen annulment tests.

Direct tests for identity of serum McDou and serum Tan with other precipitating sera and with each other.

Serum McDou, diluted 1 in 2 was compared with other precipitating sera, using a thyrotoxic thyroid extract as antigen, as described on pp. 22, 57. The precipitate produced by this serum crossed those of serum Tra (anti-SjT), serum Don (anti-SjD), and serum Dun (anti-Lup). Serum Tan, used undiluted,

formed 2 bands of precipitate, which also crossed the precipitates formed by anti-SjT, anti-SjD and anti-Lup. Serum McDou and serum Tan formed precipitates which fused completely with each other, suggesting that these 2 sera contained the same precipitating factor.

Annulment of antigen by serum McDou.

One volume of serum McDou was used to absorb 2 volumes of a thyrotoxic thyroid extract, as described on p. 146 and this treated antigen was tested with anti-SjT, anti-SjD, anti-Lup, and anti-McL, as well as with serum McDou and serum Tan. The treated antigen reacted with all these sera except serum McDou and serum Tan.

Annulment of antigen by serum Tan.

One volume of serum Tan was mixed with one volume of a thyrotoxic thyroid extract and tested as an antigen with anti-SjT, anti-SjD, anti-Lup, and anti-McL and with serum McDou and serum Tan. Neither serum McDou nor serum Tan reacted with this treated extract but reactions were still observed with the other test sera.

When sera containing anti-SjT, anti-SjD, anti-Lup, and anti-McL were used to absorb thyrotoxic thyroid extract, all the treated extracts were found to react with both serum McDou and serum Tan.

The direct identity test and tests of antigen annulment thus show that the sera McDou and Tan contain a serum precipitating factor which

differs from anti-SjT, anti-SjD, anti-Lup, and anti-McL.

Immunoelectrophoresis of serum McDou and serum Tan.

Immunoelectrophoresis of serum McDou and serum Tan was performed and the precipitating factor in each serum was shown to have the electrophoretic mobility and pattern of gamma-globulin.

Ultracentrifugation was not performed on either of these sera but from the results of immunoelectrophoresis and antigen annulment experiments, it is concluded that the precipitating factor present in both sera is an antibody.

Incidence of antigen reacting with serum McDou and serum Tan in human tissue extracts.

Serum McDou was tested in agar-gel diffusion plates with extracts of spleen, normal thyroid, thyrotoxic thyroid or submandibular gland from 40 individuals. Extracts from 35 individuals reacted when first tested with serum McDou and those from the remaining 5 reacted after they had been concentrated by freeze-drying. This antigen was also shown to be present in extracts of normal leucocytes, lymphatic leukaemic leucocytes, and myeloid leukaemic leucocytes. Incubation of a thyrotoxic thyroid extract with an equal volume of an extract of normal leucocytes or myeloid leukaemic leucocytes was performed at 37°C. for 3 hours; these

mixtures still reacted strongly with serum McDou in a precipitin test.

Incidence of the antigen reacting with serum McDou and serum Tan in animal tissues.

Serum McDou and serum Tan were tested with individual extracts of liver, kidney and spleen from guinea pigs and rabbits and with separately pooled extracts of livers, kidneys and spleens from mice. Positive reactions were obtained with organ extracts from all these animals.

Reactions of deoxyribonucleoprotein and nucleic acids with serum McDou and serum Tan.

A preparation of deoxyribonucleoprotein (DNP) from an extract of human spleen was prepared by the method of Mirsky and Pollister (1946); this was tested in an agar-gel diffusion plate with serum McDou and serum Tan and was observed to react to form a precipitate with these sera.

Portions of this DNP preparation were separately treated with deoxyribonuclease (DNAase) as described by Deicher et al. (1959), and with ribonuclease (RNAase), at 37°C. for 2 hours. A control tube containing DNP diluted with saline buffered at pH6.8 was similarly incubated. These preparations were tested in agar-gel diffusion plates with serum Tan. Treatment with DNAase completely inhibited the precipitin reaction of DNP with serum Tan, whereas treatment of DNP

with RNAase or buffered saline was without effect (Fig. 14). This experiment with DNP was repeated with the same results. Treatment with a 0.25% solution of crystalline trypsin did not affect the antigenicity of the DNP.

Portions of a thyrotoxic thyroid extract were similarly treated with these enzymes. Control tubes containing untreated DNP and thyrotoxic thyroid extract were incubated alongside the enzyme-treated antigens at 37°C. for 2 hours. These antigens were then tested with serum Tan. Treatment with DNAase destroyed the antigenicity of the thyrotoxic thyroid extract but the trypsin-treated extract and the controls were still strongly positive. Ribonuclease and trypsin therefore appear to have no effect on this antigen.

Three volumes of serum Tan were mixed with one volume of commercially prepared calf thymus DNA (Nutritional Biochemicals Corporation) and used at a dilution of 100 ug/ml. in water; 3 volumes of serum Tan were also mixed with one volume of yeast ribonucleic acid (RNA) at a concentration of 100 ug/ml. and both mixtures were kept overnight at 4°C. These mixtures were then tested with DNP. Serum Tan mixed with DNA failed to react with DNP whereas treatment with RNA had no effect on its precipitin reaction with DNP. DNA at a concentration of 100 ug/ml. was tested with serum Tan and formed a precipitate which appeared very close to the antigen well. Another portion of this DNA solution was submitted to

disintegration by sonic vibration in an atmosphere of hydrogen (Seligmann, 1958) and then titrated with undiluted serum Tan; it now reacted to form a well-defined precipitate approximately midway between serum and DNA-containing wells, at concentrations as low as 6 ug/ml.

DNA was prepared by the method of Kay, Simmons and Dounce (1952) from spleen taken after death from patient Tan. This DNA was redissolved in water at a concentration of 100 ug/ml. and subjected to sonic disintegration. Both serum Tan and serum McDou reacted to form precipitates with this antigen.

These results suggested that the antigen reacting with these sera was DNA. In order to verify this, a highly purified preparation of calf thymus DNA (provided by Dr. Keir, Biochemistry Department, Glasgow University) was submitted to sonic disintegration under hydrogen and tested at a concentration of 50 ug/ml with serum McDou and serum Tan, with both of which it formed a precipitate. This preparation of purified DNA was tested in dilutions ranging from 100 ug/ml. to 1.5 ug/ml. with serum Tan and reacted at all concentrations although at 1.5 ug/ml. the precipitate produced was faint and very close to the antigen well. Treatment of the purified DNA at a concentration of 50 ug/ml. with deoxyribonuclease completely inhibited its precipitin reaction with serum McDou and serum Tan.

It is concluded from these results that the tissue precipitating factor which reacts with serum McDou and serum Tan is DNA.

Effect of heat on antigen for serum Tan.

Specimens of a thyrotoxic thyroid extract were heated at temperatures of 55°C., 65°C., and 75°C. for periods of 30 minutes. The antigen was unaffected by heating at each of these temperatures, reactions being as strong with the heated as with the unheated extract. Specimens of a solution of sonic-treated DNA at a concentration of 100 ug/ml., heated at the above temperatures, were reactive with serum Tan, although the reaction obtained after heating at 75°C. was fainter than with the unheated DNA. Highly purified DNA at a concentration of 100 ug/ml. was heated in boiling water for 10 minutes before testing in an agar-gel diffusion plate with serum McDou and serum Tan; both sera formed a precipitate with this boiled antigen and, as before, this reaction was fainter than with the unheated preparation. The temperatures of 75°C. or higher required to cause impairment of the antigenicity of thyroid tissue extract or DNA for the antibodies in serum McDou and serum Tan closely approach those at which heat denaturation of DNA occurs.

Precipitating factors in the serum of other patients with systemic lupus erythematosus

The immunological findings in the serum of 4 patients with systemic lupus erythematosus have already been described. In 16 other patients with this condition, serum precipitating factors to human tissue extracts were found; 12 of these factors were sufficiently strong for direct tests of identity and antigen annulment tests to be performed. Sera which reacted at 1 in 8 in the preliminary test were titrated, using twofold dilutions of serum, with thyrotoxic thyroid as antigen.

The serum titres and the results of identity and antigen annulment tests are summarised in Table 11 (p.104).

Among these sera the precipitating factors identified included anti-SjT, anti-SjD, anti-Lup and anti-DNA. No other serum was found to contain anti-McL in this series.

All specimens of serum from patients with active systemic lupus erythematosus were tested with a solution of calf thymus DNA treated by sonic vibration and used at a concentration of 25 ug/ml. In addition to the sera Tan and McDou, (pp.95-101), one other serum (serum Nob) reacted undiluted with DNA in this test; serum Nob also reacted undiluted with thyrotoxic thyroid extract. Treatment of the DNA solution with deoxyribonuclease (p.100) completely inhibited formation of a precipitate with serum Nob. Although direct tests of identity and antigen annulment

tests were not performed on this serum, these findings suggest that serum Mob may also contain precipitating anti-DNA.

Three sera (Bro, McMic and Ward) contained precipitating factors which, judging by direct tests for identity and antigen annulment tests, differed from those so far described and which have not so far been further investigated.

TABLE 11

Serum precipitating factors in 29 cases of systemic lupus erythematosus

Serum	Precipitating titre	Precipitating factors present
Barb	32	anti-SjD +(?)anti-Lup
Bro	1024	Not known †
Cam	8	anti-Lup
Dun	64	anti-Lup
Glen	16	anti-SjT + anti-SjD
Len	64	anti-Lup
Moth	64	anti-SjD
Mor	16	anti-SjD
McDou	1	anti-DNA
McL	256	anti-McL
McMic	8	Not known †
Nes	64	anti-SjD + ?
Pea	1	anti-Lup
Tan	2	anti-DNA
Ward	64	Not known †
Nob	1	anti-DNA
4 other sera	1	Unidentified
9 other sera	Nil	Nil

† These sera contained precipitating factors which differed from those previously described.

* The second precipitating factor present in this serum was rather weak but it appeared from the results of antigen annulment tests that it was possibly anti-Lup.

Precipitating factors in the serum of patients with connective tissue diseases other than Sjögren's syndrome and systemic lupus erythematosus

The precipitating factors anti-SjT, anti-SjD, anti-Lup and anti-McL were also detected among specimens of serum obtained from patients with connective tissue diseases other than systemic lupus erythematosus and Sjögren's syndrome. The results of Ouchterlony agar-gel diffusion tests in all the connective tissue disease sera is given in Table 12 (p. 109).

Progressive systemic sclerosis and dermatomyositis.

A high incidence of positive results was found in progressive systemic sclerosis (9 cases) and dermatomyositis (4 cases). Serum precipitating factors were found in the serum of 6 patients with these conditions. Multiple specimens of serum were obtained at intervals of 9 months to 2 years from 3 patients whose serum contained precipitating factors; all these specimens reacted to form precipitates and no marked rise or fall in titre was observed.

Comparison of the serum precipitating factors with each other and with anti-SjT, anti-SjD, anti-Lup and anti-McL was possible in 3 cases of progressive systemic sclerosis by direct tests for identity and antigen annulment tests. (Table 13, p. 109).

No serum was obtained from a tenth patient with progressive systemic

sclerosis, but tissue extracts prepared from organs of this individual after death did not react with anti-SjD but reacted with extracts of the tissues of other individuals. These findings have already been referred to on p.64 where it was concluded that the patient's serum probably contained anti-SjD and that the antigen SjD was probably annulled by autologous antibody.

Chronic discoid lupus erythematosus.

Serum was tested from 14 patients with chronic discoid lupus erythematosus. A precipitating factor was detected in the serum of one patient but was too weak for direct tests for identity or antigen annulment tests to be performed.

Polyarteritis nodosa.

Serum from 3 patients with this condition, confirmed by biopsy, was tested for precipitating factor to human tissue extracts but none was detected.

Lupoid hepatitis.

Active chronic hepatitis associated with a positive L.E. cell test and occurring generally in young women was termed lupoid hepatitis by Mackay, Taft and Cowling (1956). Serum was obtained from only one patient with this condition and was found to contain a precipitating factor of titre 1 in 16. Antigen annulment tests were performed and

this serum was found to contain anti-SjT and anti-SjD. No precipitin reaction was obtained between this serum and a solution of commercially prepared DNA at a concentration of 25 ug/ml which had been subjected to ultrasonic disintegration in an atmosphere of hydrogen

Rheumatoid arthritis

Serum was tested from 114 patients with rheumatoid arthritis and in 12 of these precipitating factors to human tissue extracts were detected. The serum from 5 patients reacted only when undiluted and in 7 the titre was 1 in 8 or 1 in 16. Direct tests for identity and antigen annulment tests were performed on these 7 more strongly reacting sera and in 5 the precipitating factors present were identified as shown in Table 14. (p.110).

Of the two sera which remained unidentified, one gave rather faint precipitation; the other was from a patient who had severe chronic thyroiditis as well as rheumatoid arthritis and whose serum contained precipitating antibody to thyroglobulin in addition to a precipitating factor to extracts of tissues other than thyroid.

The patient Gal, whose serum gave 2 bands of precipitate and contained anti-SjT + anti-SjD, died, and a splenic extract was prepared. This extract reacted to form precipitates with serum Tra (anti-SjT), serum Don (anti-SjD), and serum Gal, the patient's ante-mortem serum. In serum Gal, therefore, anti-SjT and anti-SjD are to be regarded as auto-antibodies since they react with the patient's own tissue extract.

(Referred to previously on p. 70, Fig. 15).

Two patients in this series, McGou and McDia, were found to contain the precipitating antibody anti-McL, which has been described in a patient with systemic lupus erythematosus (pp.87-94). In a serum specimen obtained from the patient McGou 5 months previously, no precipitating factor had been detected; this was confirmed by repeating the tests on this specimen with extracts known to contain antigen McL. This patient had been transfused with 2 pints of washed, packed red cells following the collection of the first specimen of serum and 20 weeks before collection of the second. A history of recent blood transfusions was also obtained for patients McL and McDia. It thus appears that anti-McL may arise as a result of blood transfusion. (See p. 114).

Precipitin reactions between the serum of patients with connective tissue diseases and purified thyroglobulin

All sera in the connective tissue disease series which reacted with thyrotoxic thyroid extract were tested with purified thyroglobulin solution in order to eliminate the possibility that the precipitin reaction might be a thyroglobulin-antithyroglobulin reaction. A positive result was obtained with the serum from one patient with rheumatoid arthritis (p.107). The results of precipitin tests between Sjögren sera and purified thyroglobulin have already been given on pp. 60-61 where it was stated that 3 of 48 Sjögren sera contained antithyroglobulin.

TABLE 12

Results of agar-gel diffusion tests in connective tissue diseases

	Total Number	Number Positive
Sjögren's syndrome	48	15
Systemic lupus erythematosus	29	20
Chronic discoid lupus erythematosus	14	1
Dermatomyositis and progressive systemic sclerosis	13	6
Rheumatoid arthritis	114	12
Polyarteritis nodosa	3	0
Lupoid hepatitis	1	1

TABLE 13

Serum precipitating factors in progressive systemic sclerosis

Serum	Precipitating titre	Precipitating factors present
Dev	64	anti-SjD
McDon	16	anti-Lup
Pat	64	anti-SjT + anti-SjD + ? *

* This serum contained a third unidentified precipitating factor which differed from those previously described.

TABLE 14

Serum precipitating factors in rheumatoid arthritis

Serum	Precipitating titre	Precipitating factors present
Fly	8	anti-SjT + anti-SjD
Gal	8	anti-SjT + anti-SjD
McCou	8	anti-McL
McDia	8	anti-McL
McKni	16	anti-SjD

Discussion

Connective tissue diseases.

Examples of all 4 types of precipitating autoantibodies (anti-SjT, anti-SjD, anti-Lup and anti-DNA) described in this investigation were found in patients with systemic lupus erythematosus. The fifth precipitating factor, anti-McL, believed to be an isoantibody, was also found in a patient with this condition.

Various auto-immune associations have been described in all the conditions which have been grouped together as connective tissue diseases. The strongest evidence for the occurrence of auto-immunity is in systemic lupus erythematosus. The lupus erythematosus (L.E.) factor, described by Hargraves (1949) has been shown to be an antibody to deoxyribonucleo-protein (DNP) of the cell nucleus and antibodies to DNP have been detected also by the fluorescent antibody technique (Holman and Kunkel, 1957). The incidence of antinuclear factors demonstrated by the fluorescent antibody technique is much higher than the incidence of the L.E. cell factor, partly because the fluorescent antibody technique is more sensitive in detecting antibody to DNP and partly because antibodies to nuclear constituents other than DNP are revealed by the fluorescent antibody technique but do not produce the L.E. cell phenomenon (Beck, 1961). Antibodies to deoxyribonucleic acid (DNA) have been demonstrated by

precipitation and complement fixation (Ceppellini, Polli and Celada, 1957, Robbins, Holman, Deicher and Kunkel, 1957, Seligmann, 1958, Pearson, Craddock and Simmons, 1958, Deicher, Holman and Kunkel, 1959). Complement fixing antibodies to histone and crude tissue extracts have also been described (Kunkel, Holman and Deicher, 1960, Gajdusek, 1958), and Asherson, (1959), and Deicher, Holman and Kunkel (1960) described reactions with various isolated nuclear and cytoplasmic constituents. Complex patterns of reactivity were obtained between these cellular constituents and systemic lupus erythematosus sera, complement fixation being obtained not only with nuclear extracts, but in some cases with microsomal, mitochondrial or soluble cytoplasmic constituents.

Patients with systemic lupus erythematosus have also been shown to have a proneness to develop organ- or tissue-specific antibodies, e.g. to thyroid (Hijmans, Doniach, Roitt and Holborow, 1961), red blood cells (Zoutendyk and Gear, 1950), leucocytes (Seligmann, 1958), platelets, and thromboplastin (Dameshek, 1958).

Precipitating antibodies occurring in systemic lupus erythematosus.

In the present investigation, 5 distinct precipitating antibodies have been detected in the serum of patients with systemic lupus erythematosus. It is of interest to compare these precipitating antibodies with those described by other investigators.

Anti-DNA. There is no doubt that antibodies have been demonstrated to react with DNA (e.g. by Seligmann, 1958, Ceppellini et al., 1957, Robbins et al., 1957, Deicher et al., 1957). This has been further confirmed in the present study of serum McDou and serum Tan (pp. 95-101). In this study it was possible to demonstrate, for the first time, the auto-immune nature of antibody to DNA using serum Tan and DNA prepared from autologous tissue.

Anti-Lup. The antibody anti-Lup apparently does not react with DNA or purified DNP (pp. 80-82). The corresponding antigen may be nuclear protein other than DNP or a cytoplasmic constituent which has precipitated with the crude nucleoprotein preparation found to react with this factor. Seligmann (1958) has described a precipitating factor, not anti-DNA, which reacts with normal leucocytic extracts. Anti-Lup does not appear to be identical with this antibody since it has not regularly been shown to be present in extracts of normal leucocytes. (p. 80). Evidence that it is an auto-antibody rests solely on the fact that antigen Lup has been shown to be present in the tissue extracts of all individuals tested.

Anti-SjT and anti-SjD in systemic lupus erythematosus. These auto-antibodies have already been discussed on pp. 69-75 in relation to Sjögren's syndrome. They do not react with normal leucocytes, therefore they are apparently different from the anti-leucocyte precipitating factor described by

Seligmann (1958).

Anti-McL. The fifth precipitating factor examined, anti-McL, was first found in a case of systemic lupus erythematosus. It also occurred in the serum from 2 cases of rheumatoid arthritis and was found to be present in the tissue extracts of an individual who had a long history of recurring peptic ulceration and who had received blood transfusions on at least two occasions. This factor differed from the others in that it was absent from tissue extracts of 27% of individuals tested. Accordingly, anti-McL is considered to be an iso-antibody. It was not possible to confirm its iso-immune nature by testing tissues from the individuals whose serum contained the antibody. The development of anti-McL following blood transfusion in one case (p. 108) and a history of recent transfusions in the others, strongly suggests that it develops as a result of blood transfusion. Since antigen McL was absent from serum, red blood cells and plasma, it appears likely that the antigen was in the transfused leucocytes. In view of the results obtained in tests between serum McL and extracts of both myeloid and lymphatic leukaemic leucocytes, the antigen appears to be present in lymphocytes and absent from polymorphonuclear leucocytes. It may be that in normal leucocyte preparations there were insufficient lymphocytes present to give a sufficiently strong antigenic extract to react with anti-McL.

Precipitating antibodies occurring in connective tissue diseases other than Sjögren's syndrome and systemic lupus erythematosus

Auto-antibodies to cellular tissues have also been demonstrated in other connective tissue diseases by the fluorescent antibody technique (Bardawil, Toy, Galins and Bayles, 1958), and in rheumatoid arthritis by the antiglobulin consumption test (Steffen, 1959).

Precipitating antibodies to tissue extracts were also demonstrated in this study in the serum of patients with other forms of connective tissue disease.

A particularly high incidence of positive results was obtained in the group with progressive systemic sclerosis in which the serum from 5 of 9 patients possessed precipitating antibodies. This group was also examined by the fluorescent antibody technique by Dr. J.S. Beck (1962) who found that nucleolar staining was given by the serum of 2 patients with progressive systemic sclerosis. The incidence of this anti-nucleolar factor in other forms of connective tissue disease was very low, being present in only 2 patients with Sjögren's syndrome and in one patient with systemic lupus erythematosus in this series.

Of 4 patients with dermatomyositis, 2 possessed serum precipitating factors but both were weakly reacting and could not be identified. Bardawil et al. (1958) detected anti-nuclear factor by the fluorescent antibody technique in a case of dermatomyositis.

The presence of the rheumatoid (Rose-Waaler) factor in 50-70% of patients with rheumatoid arthritis suggests that an immune mechanism is active in this condition. Moreover, 13% of patients with rheumatoid arthritis have anti-nuclear factor in their serum (Weir, Holborow and Johnson, 1961). Rheumatoid arthritis is also a common feature of Sjögren's syndrome, being present in 50-75% of patients, depending on the diagnostic criteria accepted (Shearn, 1961). In the present study, 12 of 114 patients with rheumatoid arthritis were found to possess serum precipitating factors, but of these 2 were identified as being of the anti-McL type which is considered to be an iso-antibody, in each case arising probably as a result of blood transfusion.

In chronic discoid lupus erythematosus there is little evidence of auto-antibody formation. Only one patient with this condition was found to have a precipitating factor which reacted only weakly and could not be identified.

It was of interest to find that the only serum obtained from a patient with "lupoid hepatitis" possessed a serum precipitating factor. Gajdusek (1958) reported high titres in A.I.C.F. reactions (see p. 72) with serum from patients with this condition.

SECTION III

ANTIBODY TO THYROGLOBULIN

In the previous sections, antibodies were demonstrated to cellular constituents common to a wide range of human and animal tissues. The presence of antibodies to antigens common to various tissues is well established in the connective tissue diseases. In chronic thyroiditis, on the other hand, it is equally well established that antibodies occur which react specifically with constituents of thyroid tissue.

There is, however, evidence of overlap in the occurrence of organ-specific and non-organ-specific antibodies. Thus antinuclear antibodies, frequently found in the serum of patients with systemic lupus erythematosus, have been reported to occur in a proportion of patients with severe chronic thyroiditis (White, 1959, Holborow, 1960, White, Bass and Williams, 1961, Hijmans, Doniach, Roitt and Holborow, 1961) and an increased incidence of thyroid-specific antibody was reported in patients with systemic lupus erythematosus (Hijmans et al., 1961), and in patients with rheumatoid arthritis (Buchanan, Crooks, Alexander, Koutras, Wayne and Gray, 1961). There is also evidence of overlap in the occurrence of severe chronic thyroiditis and the connective tissue diseases (Bunim, 1961, Buchanan et al., 1961). In the present series of patients with connective tissue diseases, 4 sera were found to give a positive precipitin test with

thyroglobulin, indicating a high concentration of anti-thyroglobulin. It therefore seemed of interest to determine whether a group of patients with various connective tissue diseases showed an increased incidence of antibodies to thyroglobulin.

Investigation.

The several known types of thyroid antibodies have been described on pp. 7 - 9. They all tend to occur in association with chronic thyroiditis and the more severe the thyroiditis, the more likely are antibodies to be present. They occur in various combinations and if one of them is present in high titre, then the others are likely to be present (Balfour, Doniach, Roitt and Couchman, 1961). Of the various tests available, the tanned red cell agglutination test for the detection of antithyroglobulin is by far the most sensitive and it therefore was used in this investigation.

Of the 223 patients with connective tissue diseases investigated in the previous two sections, the serum of 126 was available for examination by this technique. These tests were not performed simultaneously, but with each batch of tests positive and negative controls were included and if these were not satisfactory the results were discarded. These results have already been reported (Anderson, Goudie, Gray and Buchanan, 1961).

The test series of patients with connective tissue diseases in this section included 19 patients with systemic lupus erythematosus, 26 with Sjögren's syndrome, 13 with chronic discoid lupus erythematosus, 5 with progressive systemic sclerosis, 1 with dermatomyositis and 62 with rheumatoid arthritis. The control series, obtained for comparison with the group of connective tissue diseases, was composed of 175 hospital patients whose serum had been obtained for blood grouping; this series was composed of surgical, medical and gynaecological cases. In neither the test series nor the control series was any patient included who was known to have a history or clinical evidence of thyroid disease; no patient was excluded from either series, however, because of thyroid disease detected in the course of this investigation.

It had been shown by Hackett, Beech and Forbes, 1960, and Hill, 1961, using closely similar techniques, that in hospital patients without overt thyroid disease, the highest incidence of positive results is found in females in the older age groups. Since the test series in the present investigation was composed mainly of females in these age groups, it was necessary to choose controls comparable for age and sex with those in the test series in order that the latter would not be biassed by age and sex in favour of a higher incidence of positive results. The total number in the connective tissue disease series was therefore compared with the total number of controls. In addition, the results for each type of

connective tissue disease were compared with the results in groups of patients from among the controls, of comparable age and sex. (Tables 15 and 16).

The tanned red cell agglutination test for antithyroglobulin is described on pp. 152-154. Since it is known that "purified" thyroglobulin contains traces of gamma-globulin, the specificity of the reaction was determined by performing inhibition tests with "purified" thyroglobulin and gamma-globulin. A description of these tests is given on p. 155.

In the tanned red cell agglutination test, an initial screening test was performed with all the sera at a dilution of 1 in 16. All sera positive at this dilution were titrated in fourfold dilutions from 1 in 4 to 1 in 4096 to determine their antithyroglobulin titre.

In these tests 34 (27%) of the patients with connective tissue diseases and 21 (12%) of the control series gave a positive result. Inhibition tests were performed with these sera and all the sera were found to be inhibited by pretreatment with "purified" thyroglobulin; pretreatment with gamma-globulin did not have any effect on the agglutination reactions. It was therefore concluded that the reaction was specific for antithyroglobulin. The titres in both test and control series ranged from 16 to over 4096, the titres in the connective tissue disease series being on the whole higher than in the control series.

Two of the 3 sera from patients with Sjögren's syndrome which had

previously been found to contain precipitating antibody to thyroglobulin were included in this series. Two control sera were also found to contain precipitating antithyroglobulin: there was no clinical evidence of overt thyroid disease in these 2 patients.

Table 15

Incidence of positive T.R.C. agglutination tests in connective tissue diseases and in a control series of comparable age and sex

		Sex			Age			
		Both	Female	Male	>50	<51	Average	Range
Total connective tissue diseases	No. of cases	126	95	31	85	41	53.6	21-82
	% of cases		(75%)	(25%)	(67%)	(33%)		
	No. +ve	34	26	8	28	6		
Total control series	No. of cases	175	137	38	128	47	57.1	21-89
	% of cases		(78%)	(22%)	(73%)	(27%)		
	No. +ve	21	18	3	19	2		
Rheumatoid arthritis	No. of cases	62	44	18	48	14	55.6	38-82
	% of cases		(71%)	(29%)	(77%)	(23%)		
	No. +ve	15	10	5	13	2		
Controls	No. of cases	113	84	29	88	25	56.1	30-82
	% of cases		(74%)	(26%)	(78%)	(22%)		
	No. +ve	13	11	2	13	0		
Sjögren's syndrome	No. of cases	26	21	5	23	3	60.8	38-80
	% of cases		(80%)	(20%)	(88%)	(12%)		
	No. + ve	10	8	2	9	1		
Controls	No. of cases	143	113	30	128	15	61.5	39-89
	% of cases		(79%)	(21%)	(90%)	(10%)		
	No. + ve	19	16	3	19	0		
Systemic lupus erythematosus	No. of cases	19	17	2	9	10	47.2	21-68
	% of cases		(90%)	(10%)	(47%)	(53%)		
	No. + ve	6	5	1	4	2		
Controls	No. of cases	96	84	12	51	45	50.0	21-69
	% of cases		(88%)	(12%)	(53%)	(57%)		
	No. + ve	9	8	1	7	2		
Chronic discoid lupus erythematosus	No. of cases	13	9	4	3	10	42.1	30-60
	No. + ve	0	0	0	0	0		
Progressive systemic sclerosis	No. of cases	5	3	2	1	4	39.2	27-50
	No. + ve	1	1	0	0	1		
Dermatomyositis	No. of cases	1	1	0	1	0	59.0	
	No. +ve	1	1	0	1	0		

Table 16

Significance of the increased incidence of positive T.R.C. agglutination tests in the connective tissue disease series

		Both sexes		Females
		All ages	Over 50	All ages
Total connective tissue diseases	χ^2	10	8.7	6.5
	P	<0.01	<0.01	<0.02
Sjögren's syndrome	χ^2	8.1	6.1	5.4
	P	<0.01	<0.02	<0.05
Systemic lupus erythematosus	χ^2	5.1	not significant	not significant
	P	<0.05		
Rheumatoid arthritis	χ^2	3.9	not significant	not significant
	P	<0.05		

Discussion.

In the serum of the majority of patients with Hashimoto's thyroiditis and milder forms of chronic thyroiditis, antibodies have been detected which react specifically with thyroglobulin and other constituents of thyroid tissue. This is in contrast to the tendency, already referred to (pp. 111-112), of patients with connective tissue diseases, especially systemic lupus erythematosus, to form antibodies to various tissue constituents and organs. The present study has also demonstrated that patients with connective tissue disease have in their serum a higher incidence of antibody to thyroglobulin, as demonstrated by the sensitive tanned red cell agglutination test, than would be expected in a group of patients without connective tissue disease and of comparable age and sex.

In view of these results, it may be postulated that the development of thyroid auto-immunity may occur in one or other of the following ways. In thyroid disease, anti-immunity is most frequently directed solely against the thyroid gland. The abnormality giving rise to auto-antibody formation may therefore be inherent in the thyroid gland or in the antibody-producing system. If the abnormality is present in the thyroid gland, it is assumed that the antibody-producing system reacts in a relatively normal manner to the auto-antigenic constituents of thyroid tissue released in the course of thyroid disease. If the abnormality

is present in the antibody-producing system, it is surprising that auto-antibodies to thyroid tissue alone are produced. In the connective tissue diseases, however, a range of auto-antibodies with widely differing specificities may be detected; it may be assumed that in these conditions the defect lies in the antibody-forming system. A discussion of these possibilities in relation to the concept of immunological tolerance is given on pp. 129-137.

GENERAL DISCUSSION

GENERAL DISCUSSION

The conditions termed collectively the "connective tissue diseases" were first grouped together by Klemperer, Pollack and Baehr (1942). In their studies in systemic lupus erythematosus, they observed that the common factor to the often widespread involvement of various organs was to be found in the pathological changes occurring in the connective tissue of these organs. The most characteristic change observed in the connective tissues was fibrinoid degeneration. Although these workers grouped together rheumatic fever, rheumatoid arthritis, systemic lupus erythematosus, progressive systemic sclerosis and dermatomyositis under the heading of "diffuse collagen diseases", they emphasized that in recognising connective tissue alterations as common to this group of conditions, they did not suggest that these diseases were related aetiologically.

There is, however, evidence of overlap between the clinical and pathological features of this group of diseases, which now includes Sjögren's syndrome (Cardell and Gurling, 1954). The literature reporting the coexistence of Sjögren's syndrome with systemic lupus erythematosus, rheumatoid arthritis, rheumatic fever and scleroderma has been reviewed by Shearn (1960) and Heaton (1959) has given evidence of the clinical and pathological resemblances between Sjögren's syndrome and systemic

lupus erythematosus. Rheumatoid factor, a serum factor present in the majority of patients with rheumatoid arthritis, is present also in many patients with Sjögren's syndrome (Bunim, 1961) and in some patients with systemic lupus erythematosus; it also occurs less commonly in other connective tissue diseases. Similarly, the lupus erythematosus (L.E.) cell factor which occurs in the majority of patients with untreated systemic lupus erythematosus is also found in a small proportion of patients with unequivocal rheumatoid arthritis and in other members of the group.

Family studies have been carried out in patients with connective tissue diseases: these have frequently been observed to occur in two or more members of the same family. Reports of the familial occurrence of systemic lupus erythematosus have been reviewed by McKusick (1959) and Brunjes, Zike and Julian (1961). McKusick has also reviewed the literature reporting the familial occurrence of chronic discoid lupus erythematosus and rheumatoid arthritis. The familial association of different forms of connective tissue disease has also been reported; Hagberg, Leonhardt and Skogh (1961) reported cases of progressive systemic sclerosis and systemic lupus erythematosus in one family, and Leonhardt (1961) reported the familial association of progressive systemic sclerosis and dermatomyositis.

Serological studies among asymptomatic relatives of patients with

connective tissue diseases have also been performed. A high proportion of relatives of patients with rheumatoid arthritis have been found to possess rheumatoid factor (Ziff, Schmid, Lewis and Tanner, 1958). Anti-nuclear factors have also been found to be more prevalent among relatives of patients with systemic lupus erythematosus than among patients with other connective tissue diseases (Pollak, Mandema and Kark, 1960). Hypergammaglobulinaemia is a common finding in connective tissue diseases and Leonhardt (1957), reporting the occurrence of systemic lupus erythematosus in 3 siblings, also observed elevated gamma-globulin levels in 5 of 11 other siblings of the same family.

The overlap of the clinical and pathological features of the connective tissue diseases, already referred to, indicates that they share a common aetiological factor. The frequently reported occurrence of the various members of this group of diseases within families indicates the influence of either genetic or environmental factors. Since there is no good evidence of the aetiological influence of environmental factors and since the diseases have been found to develop in blood relations, rather than in spouses (Lawrence and Ball 1958), it seems that genetic factors are more likely to be concerned.

The immunological associations of the connective tissue diseases also show familial occurrence, being found both in the members of a family who have a connective tissue disease and also in members without

the associated clinical features. Assuming that genetic factors are concerned in the immunological phenomena, they could operate in one or both of the following ways.

(a) A genetic abnormality of metabolism might give rise to abnormal antigenic breakdown products of cells and tissues.

(b) A genetically determined abnormality in the immunity system might permit the production of antibodies which react with normal body constituents.

In order to discuss this further a consideration of the factors concerned in auto-immunization is necessary.

Theories of antibody production and immune response have been revolutionized by the concept of immunological tolerance. This concept sprang from the work of Owen (1945), who showed that in dizygotic twin pregnancies in cattle, placental vascular anastomoses linking the circulations of the two embryos permitted interchange of the primordial blood cells, and that the interchanged cells survived and resulted in chimeras in which the red blood cells of the twin continued to be produced alongside the host's red blood cells and could be distinguished by their different antigenic constitution. In these circumstances, the normal immune response to foreign, homologous red blood cells had failed to operate and the adult animal was tolerant to those of its twin.

The first case of a human chimera was reported by Dunsford, Bowley, Hutchison, Thompson, Sanger and Race (1953) in a single dizygotic twin. Human chimeras have since been reported in other cases of dizygotic twins, both of whom were still alive. (Booth, Plaut, James, Ikin, Moores, Sanger and Race, 1957, Nicholas, Jenkins and Marsh, 1957). Reciprocal skin grafting was performed on the twins reported by Booth et al. and the grafts behaved as autografts rather than homografts since they were not rejected by the hosts (Woodruff and Lennox, 1959). These dizygotic twins were therefore shown to be tolerant of each other's tissue as well as of each other's red blood cells.

Billingham, Brent and Medawar (1953) showed that immunological tolerance could be induced in foetal or newborn mice by injecting them with living cells of mice of a different, histoincompatible strain. They concluded that if the immature immunity system of an individual encounters antigens, it not only fails to produce a normal, immune response but subsequently the system when mature remains incapable of producing antibodies to the particular antigens encountered earlier, although it responds normally to other antigens. Immunological tolerance cannot readily be induced by antigens which differ widely from the normal tissue constituents and such tolerance may be only partial, and may be overridden by a subsequent, strong antigenic stimulus (Owen, 1957). It is at present believed that immunological tolerance is normally acquired to the individual's own potentially antigenic tissue and body constituents. This accounts for the normal failure of the immunity system to react to autologous tissue antigens which are capable of producing immune reactions if injected into other individuals. These observations provided the most important evidence which

led to the development by Burnet (1959) of the clonal selection theory of acquired immunity.

The clonal selection theory postulates that in the immature individual a wide range of potential antibody-producing cells are found, each being capable of reacting immunologically to a single antigen or to a group of closely related antigens. The specific reactive capacity of each type of cell is considered to be passed on to descendants of the cell. If in the immature stage of development of the immunity system a potential antibody-producing cell encounters its corresponding antigen, it loses, either completely or partially, its capacity to produce antibodies against this antigen. When the individual's immunity system is mature, the remaining clones of potential antibody-producing cells are capable of multiplying in response to their corresponding antigenic stimuli. It is not known how the encounter with antigen influences the immature immunity system to bring about the state of tolerance. It may be that any clones potentially capable of reacting with a particular antigen are rendered incapable of doing so by premature encounter with the antigen and that this change in reactivity is passed on to their descendants. A second possibility is that the potential antibody-producing clones are destroyed by the encounter with their corresponding antigens.

Auto-immune Diseases

Auto-immunization may be regarded as resulting from a failure to establish or maintain the state of complete tolerance to one or more

normal body constituents. On the basis of the clonal selection theory, there are three possibilities.

(1) Certain antigens may be inaccessible to the immature immunity system at the stage when tolerance is being established. This might account for the production of auto-antibodies to antigenic constituents of thyroid, brain, testis, etc., since at the time when it is believed that tolerance is established, these antigens may be secluded from the potential antibody-producing cells or may not yet have developed.

(2) An abnormality may occur in the immature immunity system of the individual whereby it may encounter a particular antigen or antigens but fail to become fully tolerant towards it or them.

(3) Tolerance may be acquired normally but subsequently a mutation may occur within the cells of the immunity system whereby the mutant cells have lost their tolerance to one or more antigenic body constituents and give rise to cells producing antibody which will react with normal body constituents.

Organ-specific auto-immune disease with special reference to thyroiditis

Hijmans, Doniach, Roitt and Holborow (1961) have divided auto-immune diseases into two main groups briefly described as "disturbed antigen" and "disturbed tolerance" diseases, and suggest that between these groups exists a range of auto-immune disorders which have something

in common with each group. Hashimoto's thyroiditis is considered by these workers to be representative of the "disturbed antigen" diseases. The thyroid antigens concerned in Hashimoto's thyroiditis are not normally circulating, and at the time when immunological tolerance in the individual is being established, these antigens are assumed to be inaccessible to the potential antibody-producing cells and thus tolerance is not established. In consequence, if in the mature individual the potential antibody-producing cells encounter thyroid antigen which has escaped into the circulation as a result of disease or injury to the thyroid gland, they may be stimulated to multiply and produce auto-antibodies specific for thyroid tissue. This explanation of the occurrence of auto-immunity in Hashimoto's thyroiditis therefore falls within the first of the circumstances postulated above for the production of auto-immunity. Blagg (1960) has demonstrated a higher incidence of auto-antibodies in the serum of patients with thyrotoxicosis after they have been treated with radioactive iodine, suggesting that an autoantigenic stimulus results from the release of material from the thyroid gland. On the other hand, Hjort (1961) showed that auto-immunisation occurred rarely as a result of partial thyroidectomy in patients with thyroid disease into whose serum, during the few days after operation, thyroglobulin detectable by immunological methods was being continuously released. More recently, Hjort and

Pedersen (1962) have demonstrated "free thyroglobulin" in the serum of 75% of newborn infants and 50% of their mothers; thyroid antibodies demonstrable in the serum of 3 pregnant women were shown to decrease in titre during pregnancy, suggesting an in-vivo antigen-antibody reaction. The higher incidence of "free thyroglobulin" in the infants' serum appears to indicate that it is being secreted from the foetal thyroid into the infant's circulation, crosses the placental barrier into the maternal circulation and will react with maternal thyroid antibody, if present. These findings cast some doubt on the validity of the "disturbed antigen" theory with regard to thyroid disease. These infants' sera were tested for thyroglobulin only once, at the time of delivery, but no evidence of auto-immunization was found in any of 35 mothers whose serum was tested 6 to 10 weeks after delivery. From these findings Hjort and Pedersen think it possible that thyroid auto-immune disease falls into the category of "disturbed tolerance" rather than "disturbed antigen" disease. They believe that newborn infants who have free thyroglobulin in their serum have developed tolerance to thyroglobulin and suggest that in those infants whose mother's serum possessed thyroid antibody, a reaction may take place between the foetal thyroglobulin and maternal antibody which rapidly removes thyroglobulin from the foetal circulation and, in consequence, tolerance to thyroglobulin may not be established; these might therefore be the individuals whose immunity system, when

challenged by thyroglobulin in adult life (e.g. as a result of pregnancy or thyroid disease), reacts to form antibodies to thyroglobulin.

Hall, Owen and Smart (1960) have shown that there is an apparent genetic predisposition to the formation of thyroid auto-antibodies, but Hjort and Pedersen suggest that this may be a "false" genetic predisposition and may occur in siblings because their mothers possessed circulating thyroid antibodies during pregnancy.

Other organ-specific auto-immunities (e.g. to testis, brain) have not been so thoroughly investigated as auto-immune thyroiditis and there is little direct information to indicate whether inaccessibility of antigen in the intact individual is concerned.

Non-organ-specific auto-immune disease

The occurrence in the connective tissue diseases of a wide range of antibodies is in marked contrast to that of the thyroid-specific antibodies discussed above. The four auto-antibodies which have been detected in the present investigation are not organ-specific, reacting with extracts of all human cellular tissues examined, including leucocytes, and therefore it is obvious that the corresponding antigens have a wide-spread distribution in the body. Consequently, it is likely that the immature reticuloendothelial system encounters them during the period when tolerance is normally acquired. One of the antigens in particular, DNA, is known to be present in all nuclei and its release during the

maturation of erythrocytes alone would be expected to provide opportunity for the acquisition of tolerance by the primitive immunity system. If these assumptions are correct, then the development of the four auto-antibodies could result from:-

(1) failure of the immature immunity system to acquire complete and permanent tolerance in spite of the opportunity to do so.

(2) normal acquisition of tolerance but a subsequent development in the reticuloendothelial system of mutant cells intolerant to the antigens concerned.

(3) an acquired metabolic defect whereby abnormal antigenic material is presented to the immunity system which responds by antibody production.

If the first possibility is correct, the immature immunity system must fail to acquire complete tolerance to many autologous body constituents, for in addition to the antigens concerned in the present study, other cell-specific antigens, e.g. in platelets, red blood cells and leucocytes, can give rise to auto-immunity in systemic lupus erythematosus, resulting in thrombocytopenic purpura, haemolytic anaemia and leucopenia respectively. It seems likely that individuals who eventually develop these auto-antibodies must initially have acquired some degree of tolerance, since systemic lupus erythematosus and other conditions associated with auto-immunity usually occur in adults.

As regards the second possibility, the proliferation of one mutant

cell type in the reticuloendothelial system would be expected, according to the clonal selection theory, to give rise to cells producing antibody which would react with only one antigen or with a group of very closely-related antigens. In order to produce the variety of auto-antibodies found in the connective tissue diseases, several mutant cells would be required to proliferate. This situation, therefore, is considered more likely to occur in the multiple mutations concerned in lympho-reticular neoplasia than in the connective tissue diseases.

Different explanations have been put forward by various writers (e.g. by Lack, 1961, Potter, 1961) for the mechanism postulated in the third possibility. Both these writers, however, suggest that if free, noxious enzyme should escape into the tissues, it may react to give abnormal metabolic products, some of which could behave as auto-antigens. If this is correct, then it must be assumed that the four auto-antigens described in this study do not normally escape from tissues <u>in-vivo</u> except in the presence of the postulated enzyme release. These auto-antigens cannot be considered to be abnormal since they have been found in all normal cellular tissue extracts tested.

These possibilities provide a tentative explanation for the occurrence of auto-antibodies in disease. With the exception of the antibodies produced against blood constituents, however, circulating

auto-antibodies have not been shown to be the cause of the lesions in auto-immune diseases. For example, in various forms of thyroid disease auto-antibodies may frequently be detected but only rarely do the thyroid lesions progress to give the histological picture of severe chronic thyroiditis (Roitt and Doniach, 1960); the course of systemic lupus erythematosus is frequently episodic and this condition may regress completely even when auto-antibodies persist in the serum (Leading article, Brit. Med. J., 1960). Moreover, the L.E. cell factor has been detected in the serum of newborn infants of women with systemic lupus erythematosus and yet the infants have been clinically healthy (Berlyne, Short and Vickers, 1957). In the experimental auto-allergic diseases (thyroiditis, encephalomyelitis, etc.,), injection of the antigen combined with Freund's adjuvant gives rise to lesions in the corresponding organ and to circulating auto-antibodies, but in general the extent of the organ lesions has not correlated well with the titre of circulating auto-antibody. The experimental auto-allergic diseases do not resemble the corresponding human disorders in that they show regression of the disease after the action of the antigen incorporated in Freund's adjuvant has declined, whereas in man progressive tissue destruction is a feature of the disease. Passive transfer of experimental allergic encephalomyelitis has been performed in rats using lymph node cells from immunized donors which were transferred to tolerant hosts (Paterson, 1960), and

thyroiditis has also been passively transferred in guinea pigs by the same method (Felix-Davies and Wakeman, 1961). Passive transfer experiments using serum from immunized animals have not been successful in producing the experimental auto-allergic diseases. McMaster, Lerner and Exum (1961) studied the relationship of circulating antibody to experimental allergic thyroiditis in histocompatible guinea pigs and found that the antibody titre did not always correlate with the degree of thyroiditis, but that all animals with thyroiditis which were skin-tested with thyroid extract exhibited delayed hypersensitivity. These results indicate that a delayed hypersensitivity mechanism rather than circulating antibody, or perhaps a combination of both, is implicated in the tissue destruction occurring in the experimental auto-allergic diseases. In the present investigation, the auto-antibodies, with the possible exception of anti-DNA, do not appear to correlate with the clinical state of the patient. Anti-DNA was found in the serum of only 3 patients with systemic lupus erythematosus and 2 of these have since died; with such a small number it is not possible to say what significance the presence of anti-DNA may have on the outcome of the disease. The auto-antibodies anti-SjT, anti-SjD and anti-Lup have no known clinical or aetiological significance, nevertheless their occurrence in various members of the connective tissue diseases focuses further attention on the clinical and pathological resemblances occurring in these conditions and may indicate that very similar mechanisms are operative

in their pathogenesis.

The observations made in this study raise a number of problems and their solution requires much further investigation. Apart from DNA, none of the auto-antigens have been characterised; preliminary work suggests that this may prove difficult since the antigens are present in very low concentrations in tissue extracts of great chemical complexity and are rather unstable, particularly antigen SjD.

The antibodies described may provide useful tools for cytology and the antigenic cellular constituents with which they react may be concerned not only in auto-immunity, but also in the important subject of homograft immunity.

In addition to the auto-antibodies which have been described, other serum precipitating factors have been detected but remain to be investigated and it seems certain that a large number of precipitating factors will eventually be demonstrated.

MATERIALS AND METHODS

MATERIALS AND METHODS

Serum

Specimens of clotted blood were provided by clinicians of the Western Infirmary, Glasgow, and several other hospitals, from patients with various connective tissue diseases in whom the diagnosis had been reasonably established. The serum was pipetted from these specimens, centrifuged to remove red blood cells, then stored at -20°C. until required for use. Serum obtained for blood grouping and cross-matching was also provided by the Haematology Department, Western Infirmary, from hospital patients with various surgical and medical conditions, but with no clinical history of symptoms of connective tissue diseases; serum from this group of patients served as controls.

Tissue extracts.

Human tissues were generally obtained a few hours after death, or, as in the case of thyrotoxic thyroid gland, were removed surgically. Animal tissues were removed immediately after death.

Tissue extracts of thyroid, liver, spleen, etc., were prepared by mincing the organ, then homogenising in an M.S.E. homogeniser for 3 minutes at full speed with an equal volume of 0.9% sodium chloride solution (saline solution) containing one fifth volume of isotonic phosphate buffer of pH 7.5. The tissue homogenate was then centrifuged

at 2000 g. for 30 minutes and the supernate was removed and stored undiluted at -20°C. until required for use. The preparation of tissue extracts was carried out at 0-5°C. throughout.

Leucocyte extracts

Twenty ml. of venous blood from normal individuals or leukaemic patients was taken into 2 ml. of a 1.5% solution of disodium ethylene diamine tetra-acetic acid (E.D.T.A.) as anticoagulant and mixed gently by inversion of the container. 4 ml. of a 6% solution of dextran in 0.9% saline solution was mixed with the blood; the mixture was allowed to sediment for 45 minutes and the leucocyte-rich plasma was carefully removed by pipette. Up to this stage the method used was essentially that described by Walford (1960). The leucocytes were deposited by centrifugation at 900 g. for 15 minutes, washed three times in 0.9% saline solution buffered at pH 7.5, and resuspended in an equal volume of saline solution. The leucocytes were then disrupted by thrice freezing at -20°C. and thawing. After this treatment the material was used as antigen, without further centrifugation, in Ouchterlony agar-gel diffusion tests.

Precipitin Tests

In the first instance, the sera for investigation were tested for antibody to tissue constituents by a technique of double diffusion in an

agar gel (Ouchterlony, 1953). Antigen and antibody are placed in separate wells in the gel and allowed to diffuse into the gel; precipitating antibody, if present in the serum, diffuses from the serum well and encounters antigen, diffusing from the tissue extract well; where the relative proportions of antigen and antibody permit, a band of precipitate is formed in the gel.

Ouchterlony technique.

Preparation of agar plates. It was found that a 1.7% concentration of Difco 'Bacto' agar in 0.9% saline solution gave a reasonably clear gel. Sodium azide, at a final concentration of 0.1% was added to the melted agar to prevent bacterial growth and 20 ml. of this agar was poured into a 9 cm. diameter petri dish and allowed to set. In order to provide wells for the serum and antigen extract, holes were cut in the agar by means of cork-borers. Preliminary tests were performed by placing serum and tissue extract in wells of 6 mm. diameter with centres 12 mm. apart, but it was subsequently found that the sensitivity of the test was increased by using larger wells, of 10 mm. diameter, with centres 15 mm. apart; one was filled with tissue extract and the other with serum. The positioning of the wells was first of all marked on graph paper, which was then placed below the petri dish to act as a guide when cutting the wells. The agar discs thus punched out were removed by suction pipette.

For routine testing of the serum for precipitating factors, the serum was placed in wells diametrically opposite on either side of the antigen well; the serum was used undiluted in one well and diluted 1 in 8 in the other; the latter serum dilution was used since it sometimes happened that undiluted serum containing a high antibody concentration produced a precipitate which appeared so close to the antigen well that it was obscured by the opaque zone surrounding this well; at a dilution of 1 in 8, however, the precipitate appeared in the clear zone between the tissue extract and serum wells. The petri dishes were kept uncovered for a few hours after the wells had been filled, since it was found that diffusion of the reagents through the gel was hastened when evaporation also took place from the surface of the gel. A positive result showed as one or more white bands of precipitate in the agar developing within 72 hours, usually within 24 hours.

Staining technique for protein precipitated in agar-gel diffusion plates.

Occasionally it was desirable to wash the excess protein from a plate containing a weakly positive precipitin test and to stain the precipitate remaining in the agar-gel with a protein-specific dye, naphthalene black. After this treatment the result was more easily read; these tests could also be preserved for reference, since photography of weakly positive results was difficult.

Preparation of dye solution. The solvent consisted of a mixture of methyl alcohol, distilled water and acetic acid in the ratio 50:50:10 by volume. A saturated solution of naphthalene black 10B (G.T. Gurr, Ltd.) was prepared in this solvent.

Method. The agar plate was washed for at least 24 hours in several changes of saline solution and rinsed in water for 10 minutes to remove most of the salt; the agar gel was carefully transferred to a glass slide of suitable size and dried on to the slide by heating in an incubator for a few hours at 37°C. The slide was then immersed in the dye solution for approximately 30 minutes and washed in 3 changes of fresh solvent until all traces of diffuse staining of the agar were removed, the slide was then rinsed in water, dried at room temperature and preserved by painting with a coating of clear varnish ('Ercalene', Canning and Co.)

Annulment of antigen.

Principle. The precipitating factors in positive sera were compared by means of treatment of the tissue extract or constituent with a precipitating serum to annul the corresponding antigen. The tissue extract thus annulled for one type of precipitating serum continues to act as antigen with other sera containing precipitating antibodies directed against other tissue constituents which remain unblocked. Precipitating sera containing the same type of antibody as the serum used to treat the tissue extract also fail to react with the annulled tissue extract.

Technique. Tissue extract was mixed with serum containing a precipitating antibody of titre exceeding 1 in 64 in the ratio of 9 parts of extract to one part of serum; a correspondingly higher proportion of serum was mixed with the extract if the titre of the precipitating antibody was 1 in 64 or less. The tissue-extract-serum mixtures were kept at 4°C. for 18 hours to allow annulment of antigen and were then tested as antigens against various precipitating sera by the agar-gel diffusion technique. The serum used to treat the tissue extract was also tested and a negative result with this serum indicated that the corresponding antigen had been annulled. In controls performed simultaneously, the same sera were tested with the tissue extract treated with normal serum

to ensure that treatment of the extract with normal serum had not abolished the reactivity of the extract in precipitin tests.

Immunoelectrophoresis

Immunoelectrophoresis in agar was performed by a micro-technique described by Goudie (1960). By electrophoresis of the serum containing precipitating factor to human tissue extract, the fraction of serum containing the precipitating factor may be identified by placing a trough containing the tissue extract alongside the electrophoretic run and observing the position at which a band of precipitate is formed and comparing it with the bands produced between the serum and rabbit anti-human serum (Coombs reagent).

Technique.

Borate buffer. Boric acid H_3BO_3, 24.81g./litre (0.4 M.)

Sodium borate $Na_2B_4O_7 10H_2O$, 38.22g./litre (0.1M.)

These solutions were mixed in the proportion of 675 ml. 0.4 M. boric acid to 300 ml. 0.1 M. sodium borate and distilled water was added to a total volume of one litre; the pH of this buffer was 8.1.

Difco 'Bacto' agar was dissolved in borate buffer to a concentration of 1.7% and the melted agar was poured on to glass slides 8.25 cm. square which had been coated previously with 0.1% agar in distilled water and

dried in air; coating with a thin layer of dilute agar provided a better surface for adhesion of the thicker layer of agar gel (2.5 mm. approx). When the agar had set, a well 4 mm. in diameter was cut in the agar: troughs, 4 mm. in width and 7 cm. long were cut in the agar on either side of the well and in the same direction as the electrophoretic run would take place. (See fig. 4).

The serum for electrophoresis was placed in the well and electrophoresis was carried out for 2 hours at 110 volts (current approximately 20 milliamps.) for good separation of serum proteins with little rise in temperature. The troughs were then filled with tissue extract (or rabbit anti-human serum) and the plates were kept in a moist atmosphere at room temperature, inspected daily for 4 days, and the results recorded. Finally, the plates were washed and stained with naphthalene black as described on p. 145.

Complement fixation test.

Sera. The sera to be tested were heated in a water-bath at 56°C. for 30 minutes immediately before use to inactivate complement.

Complement. The source of complement was fresh guinea pig serum which was stored at -20°C. until required for use.

Indicator for complement. A 3% suspension of sheep red blood cells sensitized with 4 minimum haemolytic doses (4M.H.D.) of an immune serum from a horse injected with sheep cells (supplied by Burroughs Wellcome and Co.) was used as the indicator for the detection of complement.

Titration of complement. Complement was titrated each day immediately before use. A 1 in 5 dilution of complement was made and 0.5 ml. of sensitized sheep cells was added to a row of tubes (7.6 x 1.25 cm.) containing 0.025, 0.03, 0.035, 0.04, 0.045, 0.05 and 0.055 ml. of complement dilution respectively; the tubes were incubated in a water bath at 37°C. for one hour. The smallest volume of guinea pig complement giving complete haemolysis of the sensitized cells was taken as 1 M.H.D. of complement.

Antigens. The tissue extracts used as antigens in the complement fixation tests were prepared in the same manner as for the Ouchterlony technique. Twofold dilutions of the extracts from 1 in 1 to 1 in 8

were tested for anticomplementary activity using 2 M.H.D. of complement in each tube and incubating the tubes at 37°C. for one hour before adding 0.5 ml. of sensitized cells and incubating for a further hour at 37°C. Each extract was used in the test at the highest concentration at which no anticomplementary activity was observed, i.e. the highest concentration at which they did not fix more than 1 M.H.D. of complement. When sera had been found which fixed complement with extracts of this type, these sera were used to determine the effectiveness as antigens of fresh tissue extracts; sera of known titre were also included with each batch of tests as a check on the continuing potency of the antigenic extracts.

Test. The test for complement-fixing antibodies in serum was set up as follows.

For each serum to be investigated, 0.1 ml. volumes of undiluted serum and serum diluted 1 in 4 were used; 0.1 ml. of antigen dilution and 0.1 ml. of complement containing 3 M.H.D. was added to each tube containing serum. Anticomplementary controls for each serum and for the antigen used in the test were also included; these consisted of 0.1 ml. volumes of serum or antigen dilution and 0.1 ml. of diluted guinea pig serum containing 2 M.H.D. The tubes were shaken to mix the contents and incubated at 37°C for one hour with occasional shaking,

after which 0.5 ml. of sensitized cells were added to each tube. The contents of the tubes were again mixed and allowed to remain at 37°C. for a further hour, and the results were read visually according to the amount of lysis in each tube. Complete lysis in the 2 control tubes indicated that neither serum nor antigen alone had an anticomplementary activity of more than 1 M.H.D.; accordingly, partial or complete inhibition of lysis in the test, using 3 M.H.D. of complement, was not attributable to the sum of the anticomplementary activities of serum and antigen. Any slight inhibition of lysis occurring in the test with the undiluted serum was regarded as an equivocal (±) reaction; marked inhibition of lysis in the test containing undiluted serum was termed weakly positive (+); sera giving partial or complete inhibition of lysis in both the test containing undiluted serum and that containing the serum diluted 1 in 4 were termed strongly positive complement fixing sera (++). Positive results were accepted as indicating the presence or absence of antibody only when complete lysis occurred in the serum and antigen anticomplementary control tubes.

Tanned Red Cell Agglutination Technique

Red Blood Cells: Group O human blood, obtained by finger prick, was collected into a solution of five parts of 0.9% saline solution and one part of 3.8% sodium citrate.

Buffered Saline: This was a solution of 0.9% saline solution containing one part per hundred of M/15 phosphate buffer, pH7.2.

Tannic Acid: A stock solution of 0.5% tannic acid in distilled water was made up freshly each week and kept at 4°C. For use, this solution was diluted in buffered saline to a final concentration of 1:15,000.

Antigen: Purified thyroglobulin, prepared by the method of Derrien et al. (1948), was used at a concentration of 2mg. /ml. in buffered saline. According to Witebsky and Rose (1956), tanned red cells coated with thyroglobulin are agglutinated more strongly by anti-thyroglobulin if the solution of thyroglobulin used to coat the tanned cells is previously immersed in a boiling water bath for 2 minutes. In practice, it was found that agglutination was considerably enhanced by this procedure and the antigen was always treated in this way, and cooled to room temperature before mixing with the "tanned" red blood cells.

Sera: The sera were inactivated by heating at 56°C. for 30 minutes before use.

Normal Rabbit Serum: This was inactivated by heating in a water bath

at 56°C. for 30 minutes, absorbed twice with an equal volume of washed, packed human group O RBCs, and stored at -15°C. A solution of buffered saline containing one part per hundred of normal rabbit serum (NRS) was used to wash and resuspend the tanned, coated red cells (TRC).

Preparation of tanned red blood cells: Human red blood cells (RBCs), thrice washed in buffered saline then resuspended to 4% were added to an equal volume of the 1:15,000 tannic acid solution. After 30 minutes at room temperature, the "tanned" RBCs were centrifuged gently, washed once with buffered saline and resuspended to give a 2% suspension in buffered saline. The cells were then mixed with an equal volume of antigen (thyroglobulin) solution in buffered saline, kept at room temperature for 45 minutes, centrifuged gently, washed twice in buffered saline containing 1 in 100 NRS and resuspended to give a 1% suspension of cells in buffered saline containing 1 in 100 NRS. Meanwhile, fourfold dilutions of the sera to be tested had been made in buffered saline and one drop of each dilution was placed in a narrow polystyrene tube (3.8 x 0.5 cms.). An equal volume of 1% TRCs was added to each tube. The tubes were then shaken to mix the contents and allowed to remain at room temperature for 1-2 hours. A positive result showed an even carpet of cells widely spread over the curved base of the tube, a corner of the carpet sometimes being rolled inwards if the cells were strongly agglutinated. If no agglutination had occurred, the cells

settled in a small button at the base of the tube. The results were most easily read in a good light, using a hand lens, by viewing the tubes from below and taking great care not to agitate the contents. (Fig. 16)

Inhibition of tanned red cell agglutination

Since it is known that the 'purified' thyroglobulin used to coat the tanned red cells in these tests contains small amounts of serum gamma-globulins and other proteins, inhibition tests were performed on all sera found to be positive by the tanned red cell agglutination test in order to determine whether this test was specific for thyroglobulin. The inhibition tests were performed by treating duplicate dilutions of a positive serum with thyroglobulin and with gamma-globulin, followed by addition of the thyroglobulin-coated tanned red cells.

The thyroglobulin preparation used to coat the tanned red cells was also used in the inhibition tests; the gamma-globulin was pooled serum gamma-globulin (prepared by the Scottish National Blood Transfusion Service). It was found that a solution of gamma-globulin containing 2 mg./ml. regularly agglutinated TRCs; this is thought to be due to the presence of a low concentration of antibody to thyroglobulin in the serum of some individuals without clinical evidence of thyroid disease. Accordingly, both gamma-globulin and thyroglobulin were used in inhibition tests at a concentration of 0.5 mg./ml., at which no agglutination occurred.

Inhibition tests were performed on positive sera which had been diluted 1 in 2 to 1 in 16 (depending on the TRC titre of the serum). One drop volumes of the serum dilution were separately mixed with one

drop of thyroglobulin solution, one drop of gamma-globulin solution, and one drop of buffered saline as control. To each mixture was added one drop of tanned, coated red cells and each was mixed again and allowed to remain at room temperature for 1 - 2 hours before reading the results.

Preparation of radioactive labelled (^{131}I) antigen or antibody

The method of labelling of protein is essentially that described by Francis, Mulligan and Wormall (1954).

An excess of iodine was shaken in 25 ml. of 0.01M potassium iodide solution on a shaking machine for a few hours at room temperature. The amount of iodine which had dissolved in this solution was estimated by titration of a portion of the filtered solution with 0.01N sodium thiosulphate solution. For every 100 mg. (approximately) of protein in the solution to be labelled, 3 mg. of free iodine was required. Accordingly, a volume of iodine-iodide solution containing the required amount of free iodine was added to a solution containing 1 millicurie ^{131}I.

15 ml. of a fresh thyrotoxic thyroid gland extract, which had been centrifuged twice at 34000 g. for 30 minutes, was made alkaline with 1.5 ml. of 5N ammonia and the radioactive solution was added, drop by drop. The solution was allowed to stand for a few minutes while iodination was completed and the solution was then adjusted to pH 7.3 with 2N. acetic acid. Any ^{131}I remaining uncombined with protein in this solution was removed by passing the solution through an 'Amberlite' column.

A similar procedure was followed to prepare ^{131}I labelled serum.

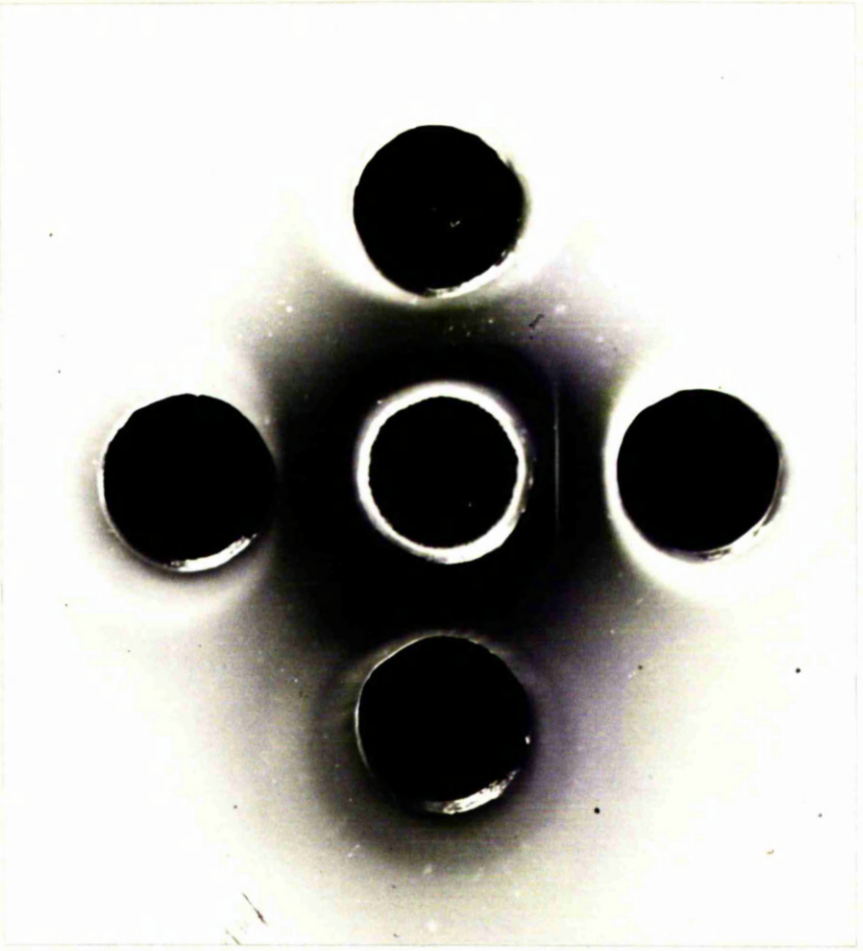


Fig. 1

Precipitate formed between serum from a patient with Sjögren's syndrome (serum Tra in well on right) and an extract of parotid gland (centre well). The other wells contain non-reacting serum. (x 2)

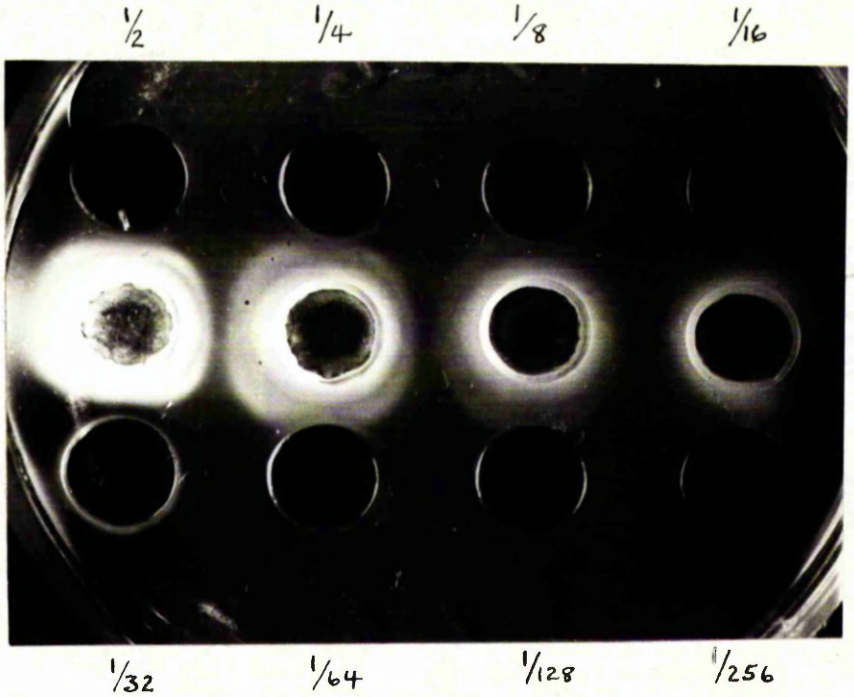


Fig. 2.

Twofold dilutions of Sjögren ~~syndrome~~ serum (upper and lower wells) and an extract of human liver (centre wells). The precipitates produced are obscured by the dense zones of opacity round the tissue extract wells but can be seen with the first three serum dilutions. The apparent diminution in the opacity round the tissue extract wells towards the right of the photograph is due to a lighting effect and not to dilution of the antigen extract (x 1)

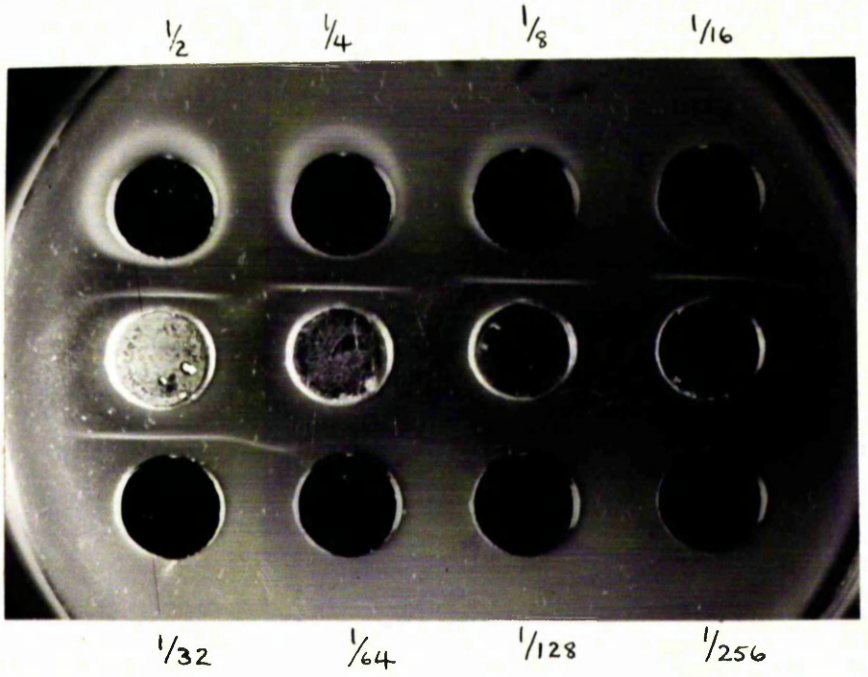


Fig. 3A

Twofold dilutions of serum Tra reacting with an extract of thyrotoxic thyroid gland. The initial dilution (upper left well) is 1 in 2 and increasing dilutions are placed from left to right along the upper and lower rows. Undiluted tissue extract is in the centre row. Reactions are visible up to a serum dilution of 1 in 64. (x 1)

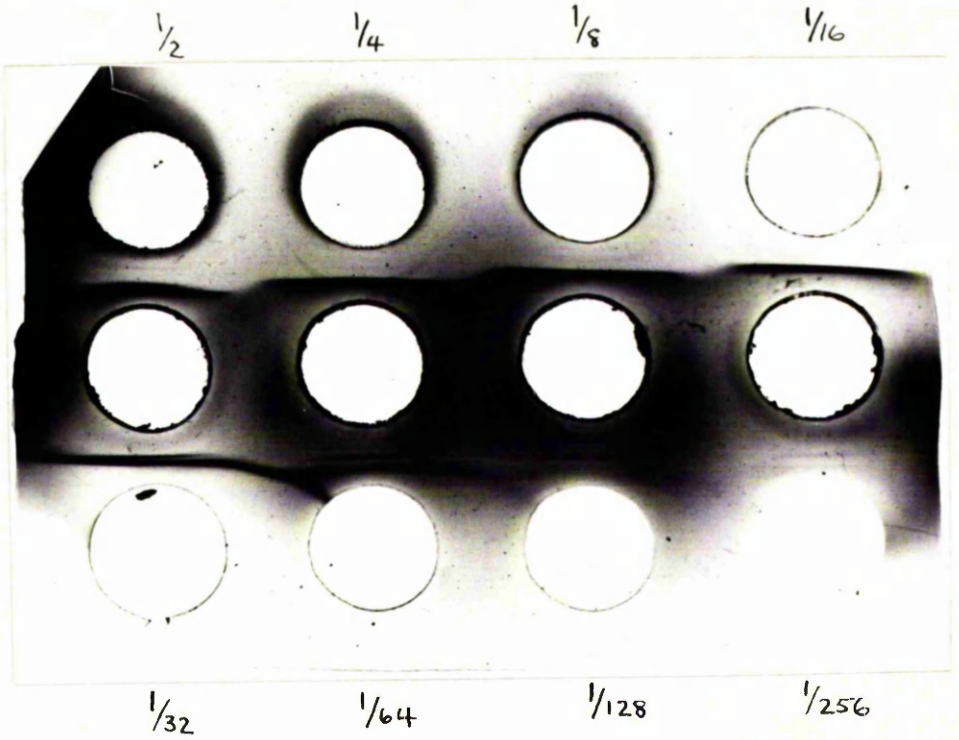


Fig. 3B

This is the same plate as 3A after staining with naphthalene black. Removal of excess protein from the plate by washing and subsequent staining of the precipitate has made it possible to see that serum Tra reacts up to a dilution of 1 in 256. The reactions of serum Tra at higher dilutions are clearly complex and a second antibody, visible only in the stained plate, may be present. (x 1)

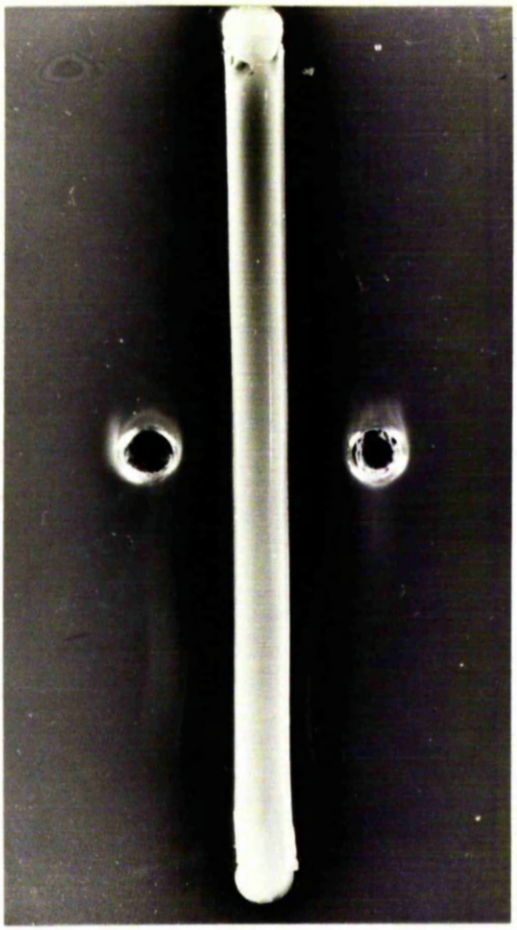


Fig. 4

Immunoelectrophoresis of serum Tra (right well). Immunoelectrophoresis of a serum containing thyroglobulin precipitin, present in the gamma-globulin serum fraction is also shown (left well). The factor in serum Tra reacting with thyrotoxic thyroid extract therefore corresponds electrophoretically to gamma-globulin. (The antigen reacting with the thyroglobulin precipitin is in excess and the precipitate formed has migrated, appearing broad and fuzzy). (x 1½)

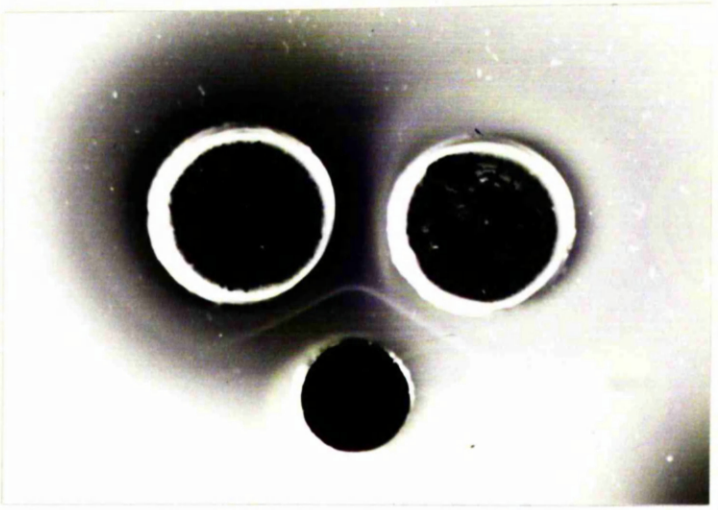


Fig. 5

Reaction of identity of the precipitates formed with serum Tra (small well) and in extracts of thyrotoxic thyroid gland (large well, left) and lymphatic leukaemic leucocytes (large well, right). (x 2)

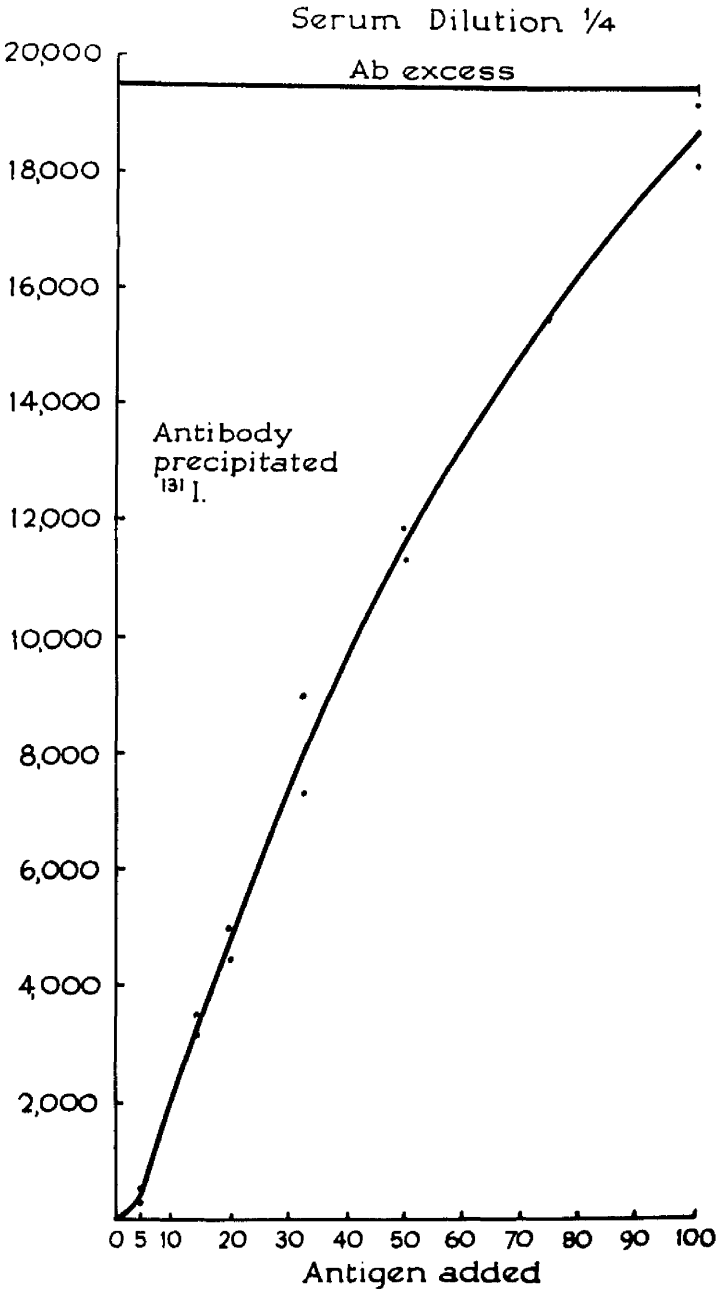


Fig. 6

Quantitative precipitin curve between ^{131}I-labelled serum Tra diluted 1 in 4 and serial dilutions of a pooled extract of thyrotoxic thyroid gland.

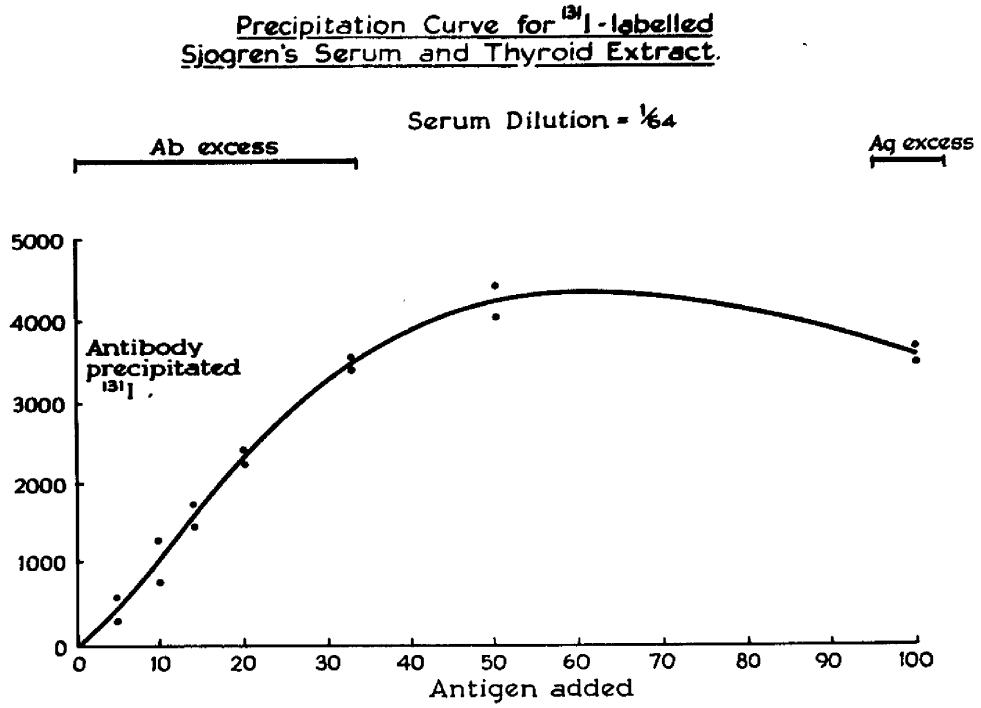


Fig. 7

Quantitative precipitin curve between ^{131}I-labelled serum Tra diluted 1 in 64 and serial dilutions of a pooled extract of thyrotoxic thyroid gland.

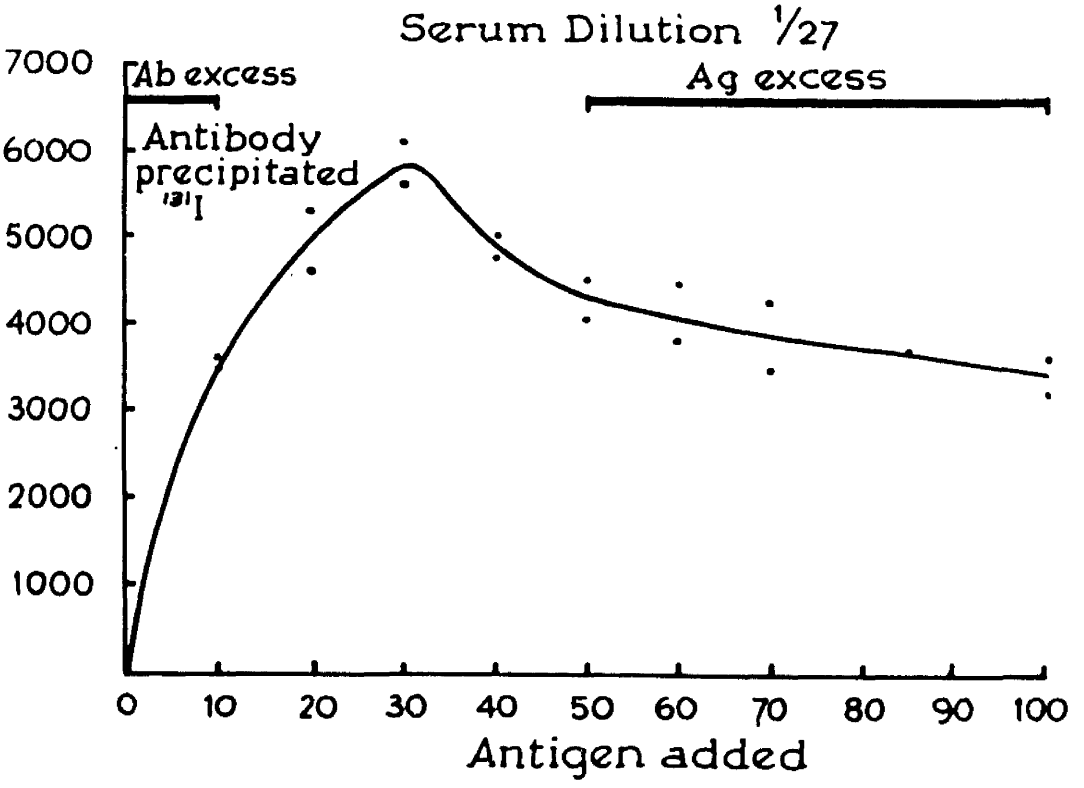


Fig. 8.

Quantitative precipitin curve between ^{131}I-labelled serum Tra diluted 1 in 27 and serial dilutions of a concentrated extract of thyrotoxic thyroid gland; 4 ml. of each dilution of extract was mixed with 1 ml. of serum dilution.

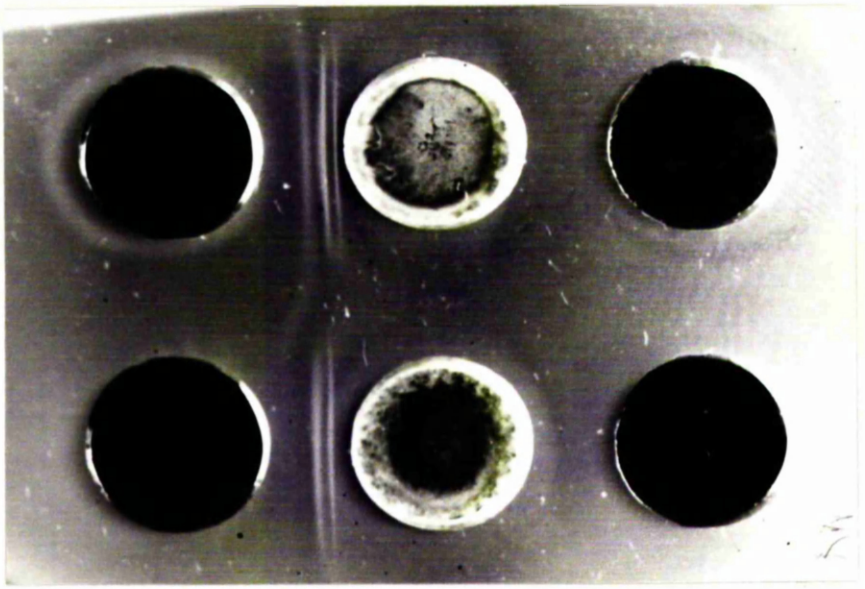


Fig. 9

Serum Scr is in the upper left well undiluted, forming 3 bands of precipitate with an extract of thyrotoxic thyroid gland (centre); in the lower left well is serum Scr diluted 1 in 8. The right hand wells contain a negative serum at the same concentrations as serum Scr. (x 2)

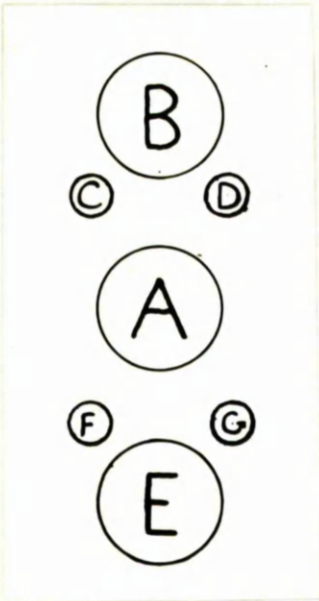


Fig. 10 A

Arrangement of wells for a direct test for identity of precipitating factors in Sjögren sera. The centre well A contains the tissue extract and on opposite sides of this well B, C and D on one side and E, F and G on the other contain the precipitating sera, which are used at an optimal dilution for the size of well and distance from the central antigen well.

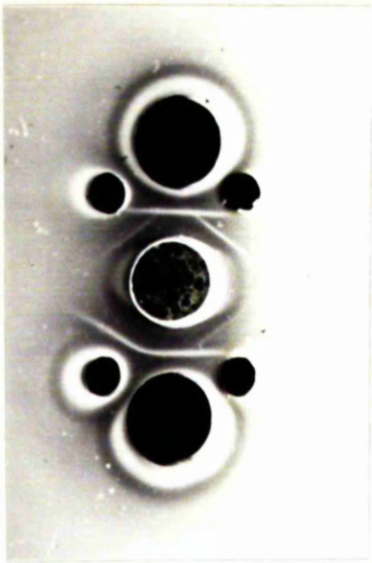


Fig. 10 B

A direct test for identity of precipitating factors in Sjögren sera. Well A contains thyrotoxic thyroid gland extract. Well B contains anti-SjT which forms a "reaction of non-identity" with both anti-SjD sera in wells C and D; wells E and F contain sera whose precipitating factors are both anti-SjD forming precipitates giving a "reaction of identity" whereas well G contains anti-SjT which forms a "reaction of non-identity" with the serum in well E. (x 1)

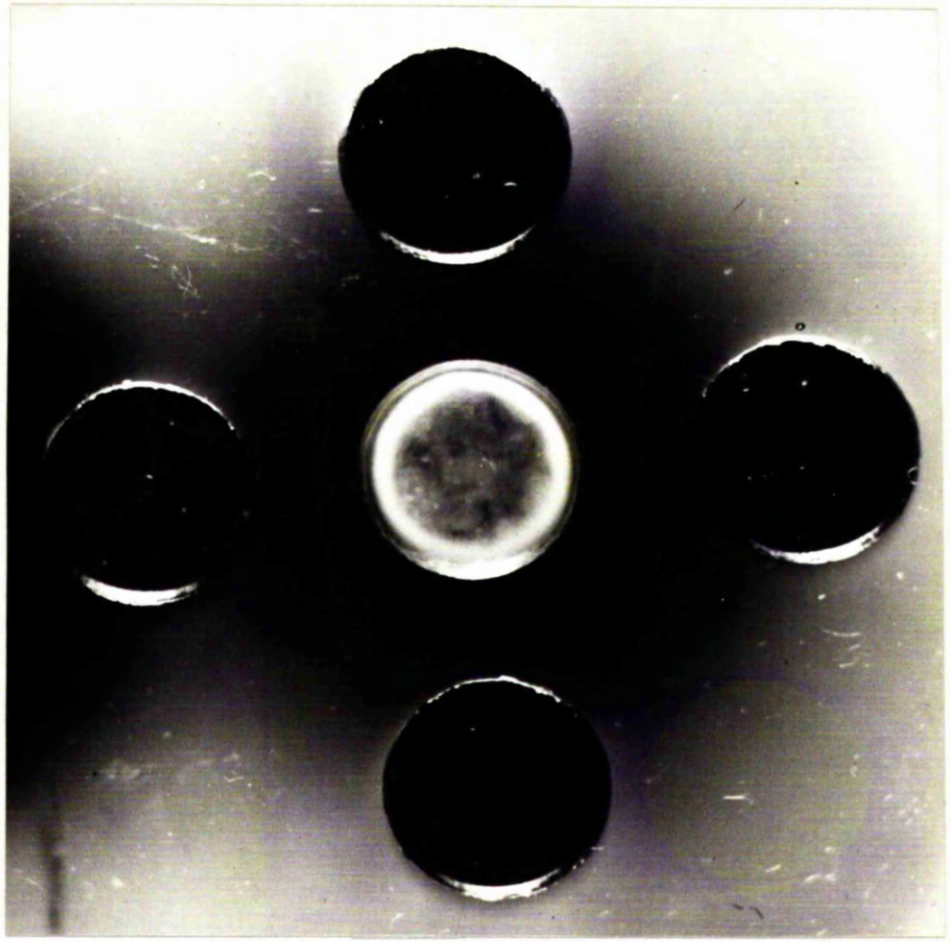


Fig. 11

Appearance of precipitates formed by anti-SjD, anti-McL and anti-Lup with an extract of thyrotoxic thyroid gland. Serum Dun (anti-Lup) is on the left and here forms 2 bands of precipitate; the upper well contains serum Don (anti-SjD) and serum McL (anti-McL) is on the right, both anti-SjD and anti-McL form precipitates which are concave towards the serum well. The lower well contains a negative serum. (x 2.5)

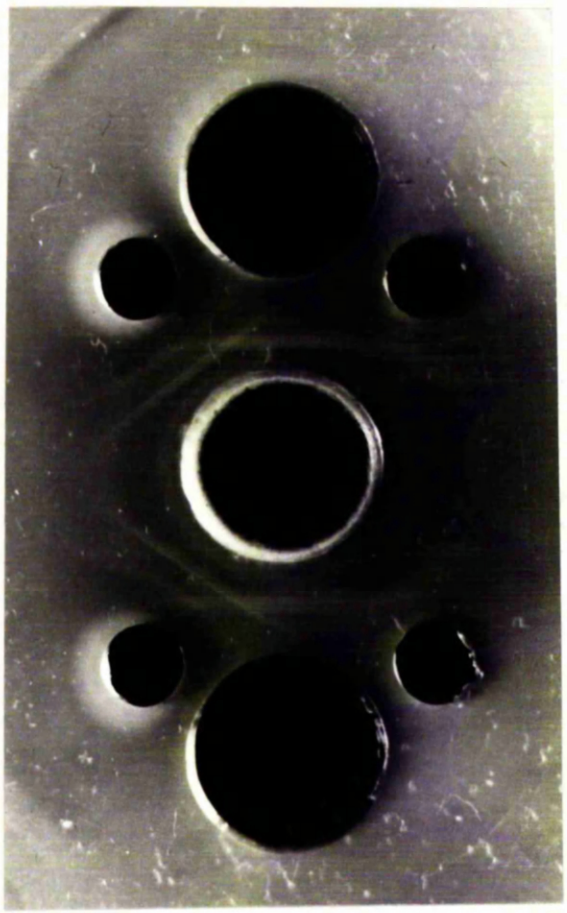


Fig. 12

Reaction of non-identity given by undiluted serum Dun (small wells on left) and serum Plu, containing anti-SjT + anti-SjD, diluted 1 in 4 in upper large well and 1 in 16 in lower large well. The small wells on the right contain serum Dun diluted 1 in 8 but insufficient antibody is present to form a distinct precipitate visible in a photograph. (x 2)

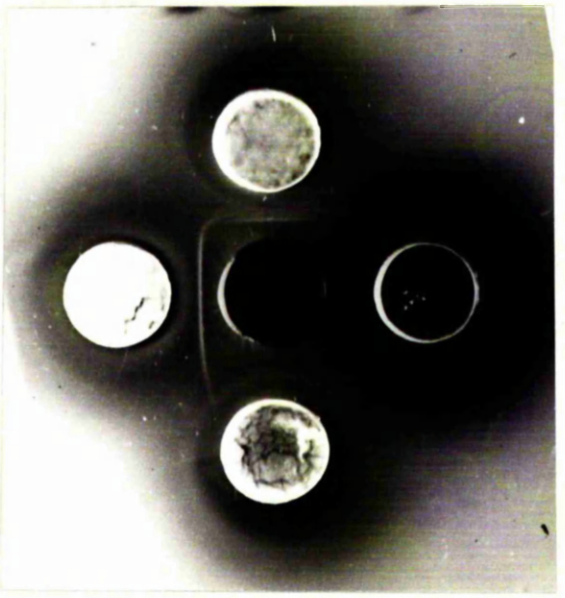


Fig. 13

Serum McL (centre well) with 4 thyrotoxic thyroid gland extracts. Well defined precipitin reactions are seen with 3 extracts but there is no reaction with the fourth (lower well). (x 1)

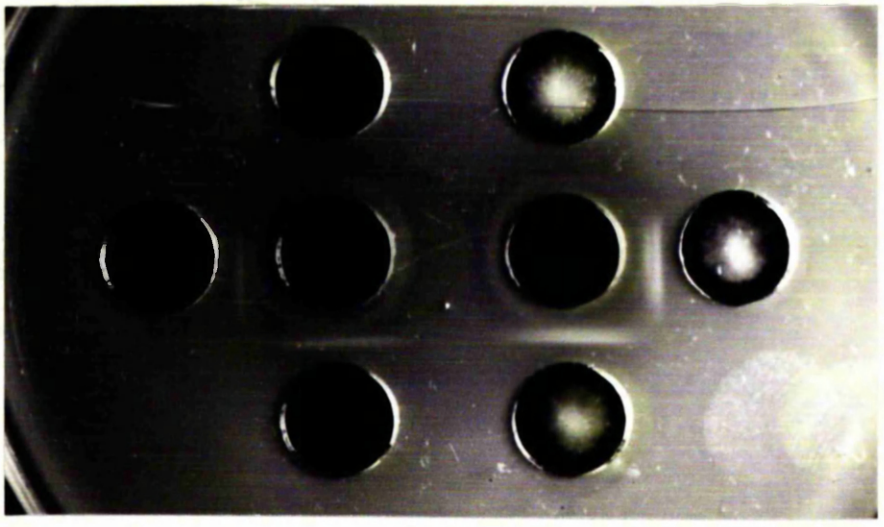


Fig. 14

Two preparations of DNP from human spleen tested with serum Tan (centre wells). Extreme left and right wells contain untreated DNP, upper wells contain DNP preparations treated with deoxyribonuclease, and lower wells contain DNP preparations treated with ribonuclease. The reaction is abolished by treating the preparation of DNP with deoxyribonuclease but not by treatment with ribonuclease. (x 1).

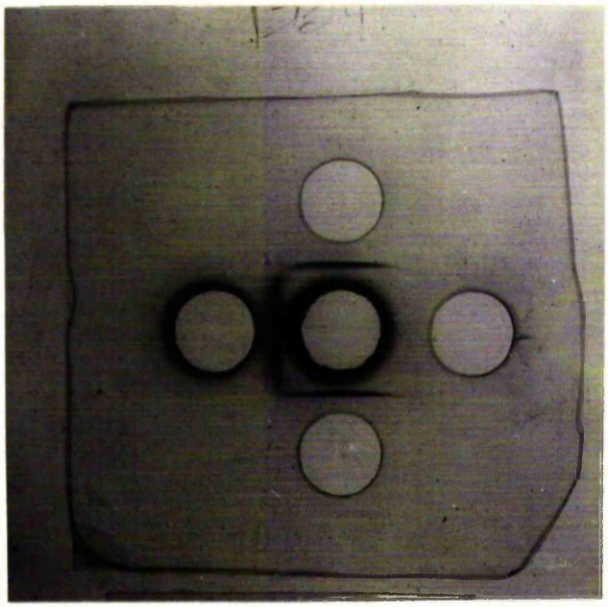


Fig. 15

Plate stained with naphthalene black showing splenic extract from patient Gal (centre well) reacting with serum Tra (upper well) containing anti-SjT serum Don (lower well) containing anti-SjD and autologous serum Gal (left) containing both anti-SjT and anti-SjD. (x 1)

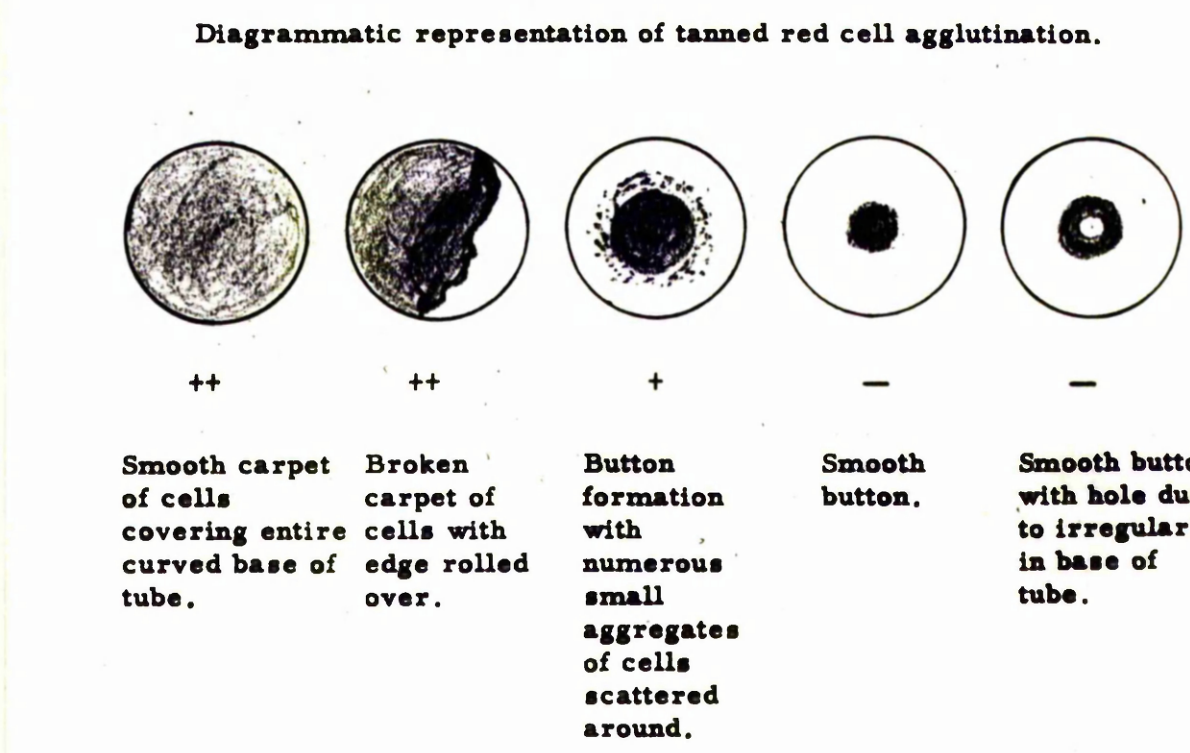


Fig. 16

Diagrammatic representation of positive and negative agglutination of tanned red cells, coated with thyroglobulin.

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